VASCULARIZATION OF THE PANCREAS AND ISLETS OF LANGERHANS IN INTRAUTERINE GROWTH RESTRICTED FETUSES AND LAMBS

By

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A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelor's degree
With Honors in

Microbiology

THE UNIVERSITY OF ARIZONA

May 2009

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Abstract

Intrauterine growth restriction (IUGR) is associated with obstructing the overall development of an organism due to nutrient deficiency, which can specifically alter the development of the fetal pancreas architecture. Therefore, development programming that impairs the pancreas can cause diabetes in adulthood of IUGR infants. The aim of this study is to assess the effect of IUGR on the pancreatic vascularity in an ovine model, comparing a control and an IUGR group, using immunofluorescent staining and morphometric analysis to measure vessel density in the pancreas and the islets of Langerhans. At 70% gestation, we found a 10.1% decrease ($P<0.05$) in vascularity of overall pancreatic tissue in IUGR fetuses compared to the control fetuses and 19.6% decrease ($P<0.05$) in the islet vessel area. At 90% gestation, the disparity increased to a 41.1% decrease ($P<0.05$) in vascularity between the control and IUGR pancreata, and a 64.6% decrease ($P<0.05$) in islet vascularity. No significant differences were found in the pancreatic or islet vascularity of IUGR or control lambs. These findings, in conjunction with previously collected data, lend to the idea that reductions in vascularity may precede declines in beta-cell mass. These impairments of pancreatic normalcy may contribute to the development of diabetes in adulthood.

Introduction

Inadequate oxygen and nutrient supply across the placenta is known to lead to intrauterine growth restriction (IUGR), which can result in a decrease in fetal size. IUGR can result from a variety of external pressures on the maternal organism, and can be emulated in the laboratory by exposing the pregnant ewe to hyperthermic conditions during pregnancy. The severity of the effects observed in IUGR fetuses range from the onset of various adulthood metabolic diseases to infant morbidity and mortality (Resnik R; 2002). One marked issue arises from lower glucose supply to the IUGR fetus' which impairs insulin secretion responsiveness (Nicolini U et al, 1989), most likely as a result of pancreatic beta-cells producing and secreting less insulin. The secretion of less insulin during fetal development lends directly to growth retardation, suggesting the beta-cells have the ability to coordinate fetal growth with the nutrient supply (Fowden AL, 1992). Moreover, in the pancreas of the IUGR sheep fetuses, decreased insulin may lead to a lower number of beta-cells because of slower mitotic rates, which was observed at 90% of gestation, and result in the reduction of beta-cell mass near term (Limesand SW; 2005). These outcomes, if persistent after birth, can lead to the individual's inability to properly process glucose in a comparably nutrient-rich diet, thus increasing the risk for developing Non-Insulin Dependent Diabetes Mellitus, commonly known as Type 2 Diabetes. Type 2 Diabetes is characterized by high blood glucose levels due to increased insulin resistance and beta-cell dysfunction. This is a disease that generally develops

later in life, but epidemiological studies indicate the risk is increased for IUGR fetuses (Bailes B; 2002).

Adequate vasculature is an integral component in the development of all organ systems of a fetus, including the pancreas. Islets of Langerhans comprise only 1% of the pancreatic tissue mass, but are highly vascularized “mini-organs” which receive between 5 and 15% of the pancreas’ blood flow. The vasculature in the pancreas also supplies a basement membrane for the beta-cells. Basement membranes are not produced directly by the beta-cells, but are responsible for contributing to the modulation of cell proliferation, differentiation, development, and repair. Rather than the beta-cells producing their own basement membranes, endothelial cells from capillaries provide a basement membrane, which promotes beta-cell function and proliferation. This vasculature formation is stimulated by the presence of the protein Vascular Endothelial Growth Factor (VEGF). VEGF contributes to the formation of new vasculature and the maintenance of existing vasculature. Limiting VEGF secretion, a proposed consequence in IUGR fetuses, causes the elimination of basement membrane components, which in turn impairs beta-cell formation and insulin expression (Nikolova G et al; 2006). The relationship between pancreatic development and vasculature is of obvious importance because it contributes to the ability of the beta-cells to proliferate and function correctly.

The specific hypothesis in this study is that decreases in production and secretion of insulin, as caused by intrauterine growth restriction, will cause a decrease in pancreatic vascularization in the fetus. This would later be followed

by reductions in beta-cell mass due to the loss of basement membrane activities. Since these three events occur between 70% and 90% gestation (based on preliminary data), we predict that reductions vascularity at 70% gestation will precede reductions in beta-cell mass, which have proven to be more prominent at 90% gestation (Limesand SW, 2005). Vascular measurements will also be collected in post-natal lambs to assess the animals' ability to adapt to a non-stressful environment. It is predicted that the vasculature and beta-cell mass will begin to normalize in IUGR lambs in an attempt to "catch up" with the control group of animals. The impact of this study will improve our understanding of factors that impair beta-cell mass, and thereby provide us with possible clinical intervention strategies to improve the outcome of beta-cell function, and ultimately lower the risk for the development of metabolic disorders, such as Type 2 Diabetes, in adulthood.

Methodology

Pancreata were collected from control and IUGR fetuses and lambs from our hyperthermia induced ovine model of intrauterine growth restriction, previously described (Limesand et al; 2006). Tissue sections from each of the groups of samples, both control and IUGR for 103 dGA (70% gestation), 135 dGA (90% gestation), and post-natal lambs (17 dPN) were obtained, frozen in OCT freeze media, and cut into 10 micrometer intervals for analysis. The tissue sections were rehydrated in water two times for 5 minutes. The pancreatic sections were microwaved in 10 mM citric acid buffer, pH 6.0, twice, for 5 minutes at 60% power. The slides were then cooled for 20 minutes and washed three times with PBS for 10 minutes. The pancreatic sections were blocked with 0.5% NEN Block for 60 minutes. The following primary antibodies were then added to the sections and allowed to incubate overnight: GP Anti-Insulin, Ms Anti-Glucagon, Rab Anti-PP, rabit anti-somatostatin, and anti-GS1 FITC. The next day the pancreatic sections were rinsed with PBS three times for 10 minutes. The following secondary antibodies were added to the sections and allowed to incubate for 60 minutes: Anti-GP AMCA Blue, Anti-Rb Texas Red, and Anti-Ms Texas Red. The pancreatic sections were then rinsed in PBS three times for 10 minutes. The slides were mounted with 50% glycerol in 10mM Tris-HCl (pH 8.0) (Limesand et al; 2005).

A Leica DM5500 microscope system was used to visualize fluorescent images differentiating between glucose, insulin, various hormones, and vasculature. GS1 demarcated the vasculature, AMCA Blue marked insulin, and

Texas Red distinguished other islet endocrine cells (alpha, delta, and F cells). These images were digitally captured with a Spot Pursuit-CCD camera, and then morphometric analysis was performed to quantify the percentage of vasculature using Image Pro 5.1 software.

To analyze the islets of Langerhans, the region of interest was outlined using endocrine staining and the positive fluorescent area for GS1 was measured relative to total islet area.

The percent vasculature positive area was determined for 15 fields of view collected randomly across each pancreatic section. The positive fluorescent area was divided by the total area of tissue. T test was performed in Microsoft Excel to determine the difference between the groups. Significance was accepted at $P<0.05$. Data are presented as the mean ± standard error of the mean.

Results

The mean total pancreas vascular area and the mean percentage of islet positive vasculature area for the fetuses at 70% and 90% of gestation as well as for the post-natal lamb pancreata were determined in this study and are shown in Table 1.

Figures 1 and 2 represent the results obtained from the fetuses at 70% gestation. At this stage of development, we found a significant 10.1% decrease in vascularity of overall pancreatic tissue of the IUGR fetuses as compared to the control group and 19.6% ($P<0.05$) decrease in the islets of Langerhans' vessel density.

Figures 3 and 4 graphically depict the results obtained from the fetuses at 90% gestation. At this stage of development, the difference in vascular disparity between control and IUGR fetuses increased from that observed at 70% of gestation to 41.1%, and a 64.6% decrease ($P<0.05$) in islet vascularity was observed.

Finally, Figures 5 and 6 show the post-natal lamb data. IUGR samples showed a 25.4% increase in overall pancreatic vascularity and 13.1% increase was observed in the islets. These values did not reach statistical significance.

Discussion

This study analyzed the effect of placental insufficiency-induced intrauterine growth restriction on pancreatic vascularity in an ovine model containing both fetal and post-natal specimens. This model organism was chosen due to its many similarities with human IUGR fetuses. The results obtained through this study indicate that IUGR decreases the vascularity in the fetal pancreas, both overall in the pancreatic tissue and more notably within the islets of Langerhans. Strikingly, the vascularity between control and IUGR pancreatic tissue and their islets was found at 70% gestation and came before an appreciable decline in the beta-cell mass was observed. The vascular area in the islets did not improve with age because at 90% gestation IUGR animals showed a 41.1% decrease from the control fetuses. These findings suggest that no progression of vasculature development occurred. Furthermore, the difference between the control and the IUGR groups for pancreatic tissue vasculature at 103 dGA was less severe (19% less) than the difference between the groups at 135 dGA. This indicates that the ability for vasculature to develop normally continually decreases as the IUGR pregnancy progresses.

Evidence for reductions in pancreatic tissue vasculature and islets' vasculature as early as 70% gestation supports our hypothesis that insulin is a major factor because insulin secretion was also blunted at this time. However an alternative hypothesis could be that the islet vasculature is regulating beta-cell mitosis, thus the initial declines in blood vessel number may contribute to later declines in beta-cell mitosis. Preliminary studies have verified that beta-cell

mass was only marginally less at 103 days gestational age in an IUGR pregnancy but significantly less at 135 dGA (Limesand SW; 2005), therefore this data confirms that the decrease in vasculature indeed occurs before the decrease in beta-cell mass but after the blunting of insulin secretion responsiveness.

The increase in vascularity in the IUGR tissues after birth could possibly be attributed to a process known as “catch up” growth. Catch up growth has been found to occur in IUGR models and human infants with regards to other metabolic processes and insulin secretion capabilities, and as found in this study, could influence (or be influenced by) pancreatic vascularity as well. Catch up growth occurs when the body adapts to low nutrients in-utero by slowing its growth but then after birth when nutrients are sufficient tries to normalize itself (De Blasio et al.; 2007). The problem that is foreseen with expedited growth is that the body may wear down due to overuse, and eventually age too quickly. This could contribute to the development of Type 2 Diabetes later in life. At a young age, the body adapts to the environment and can produce and utilize insulin just as non-growth restricted organisms; however, because of the increased workload that the body undertakes during this time, eventually the body will be unable to maintain normalcy.

To support this idea of catch up growth, it would be useful to repeat this study on an older group of lambs. Observing the amount of pancreatic vasculature in adult sheep could be beneficial to the study because these lambs were measured at 3 weeks of age. This would lend to additional insight into

whether development of pancreatic vasculature continues to develop at an increased rate, normalizes and parallels that of the control lambs, or decreases again due to overworking.

Further investigation on this topic is needed in order to define whether insulin or the vasculature is the driving mechanism for reductions in islet mitosis and mass. One topic to be looked at more closely is the role that VEGF plays in the development of the control and IUGR fetuses and lambs. The amount of VEGF present is directly correlated to the vasculature of the tissue. For this reason, less VEGF in the IUGR samples is anticipated, and would further support our current hypothesis.

Acknowledgements

I would like to thank Dr. Sean Limesand for giving me the opportunity to complete this project and for having me work in his laboratory for the past year and a half. I would also like to thank Miranda Anderson for all of her invaluable guidance in the laboratory, and Marina Martinez for her preliminary data collection for this study.

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Appendices

Table 1: Percent Vascularity of Pancreatic Tissue and Islets of Langerhans

	Total Tissue			Islets of Langerhans	
	Control	**IUGR**		**Control**	**IUGR**
103 dGA	3.338 ± 0.002	3.00 ± 0.002*		9.66 ± 0.02	7.77 ± 0.01*
135 dGA	6.558 ± 0.003	3.865 ± 0.01*		19.75 ± 0.02	6.99 ± 0.01*
Post-natal	4.193 ± 0.004	5.260 ± 0.004		11.82 ± 0.03	13.37 ± 0.03

The percentage of vascularity with the standard error of the mean for the total pancreatic tissue and the islets of Langerhans for the various groups of interest are presented. This was determined capturing digital images of fluorescently stained tissue, calculating the total area, followed by determining the amount of vasculature (fluoresced green with GS1 FITC). The standard error of the mean was calculated using six animals for each group.

* Represent significant data points ($P<0.05$).

Fig. 1 103 dGA Pancreatic Tissue

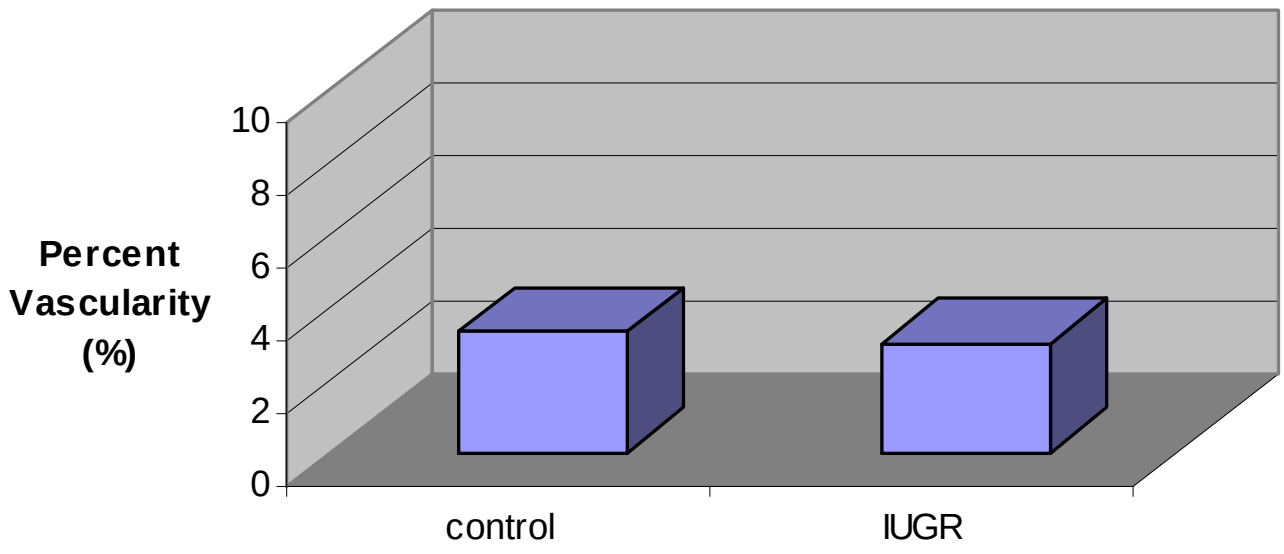


Fig. 2 103 dGA Pancreatic Islets

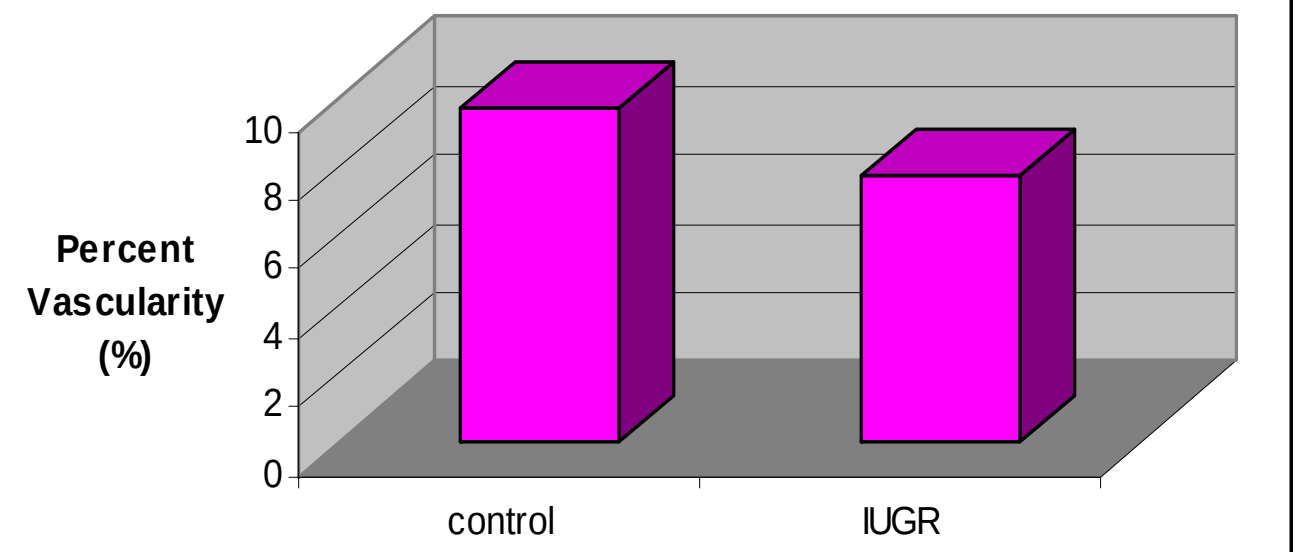
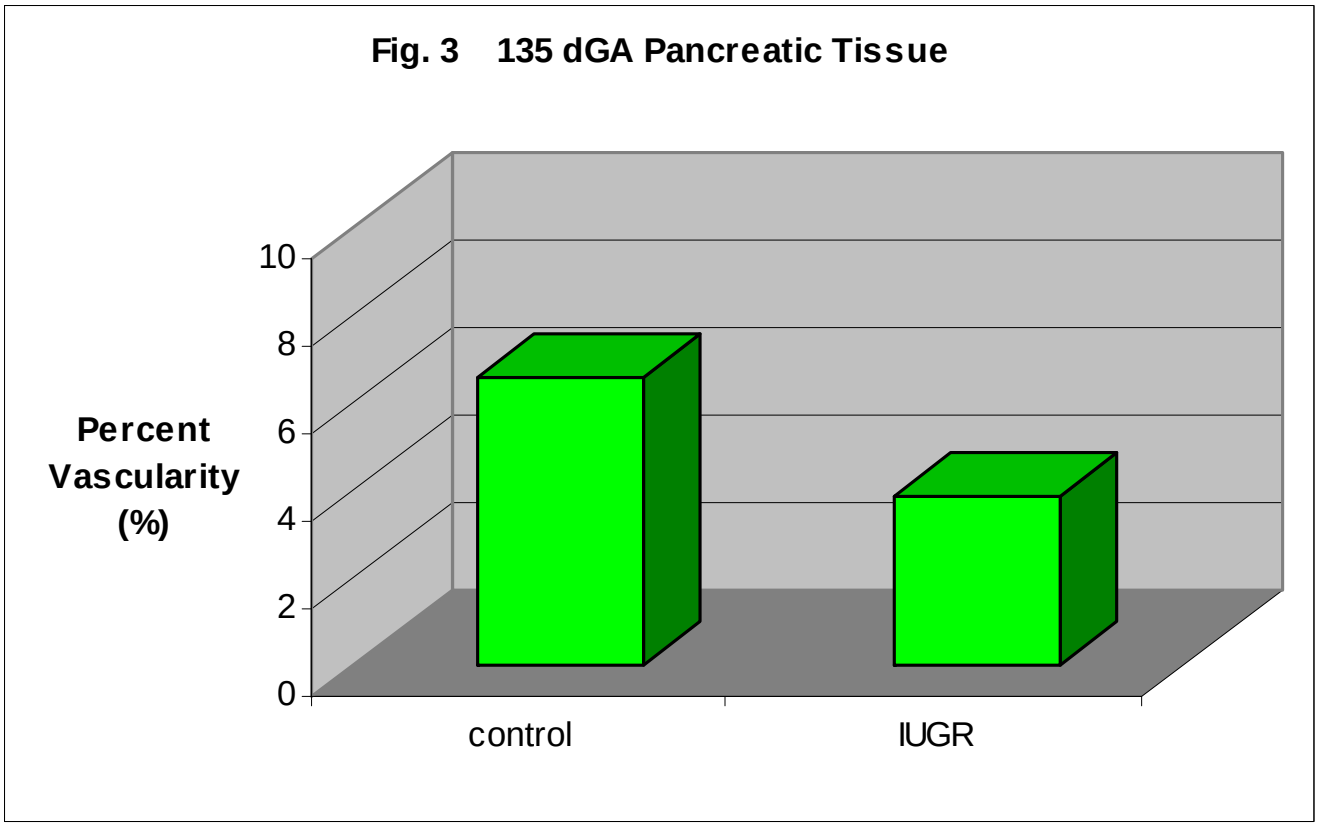


Fig. 1 & 2 represent the percent vascularity of overall pancreas tissue (Fig.1) and pancreatic islets of Langerhans (Fig. 2) in fetal sheep at 70% gestation (103 dGA). Images of the tissue were obtained and the overall area of the image was determined. The area of vasculature was determined by staining the vasculature with GS1 FITC, which caused the vasculature to fluoresce in green (see Figs. 7-9). The percent vascularity was then measured and expressed relative to total pancreatic or islet area.



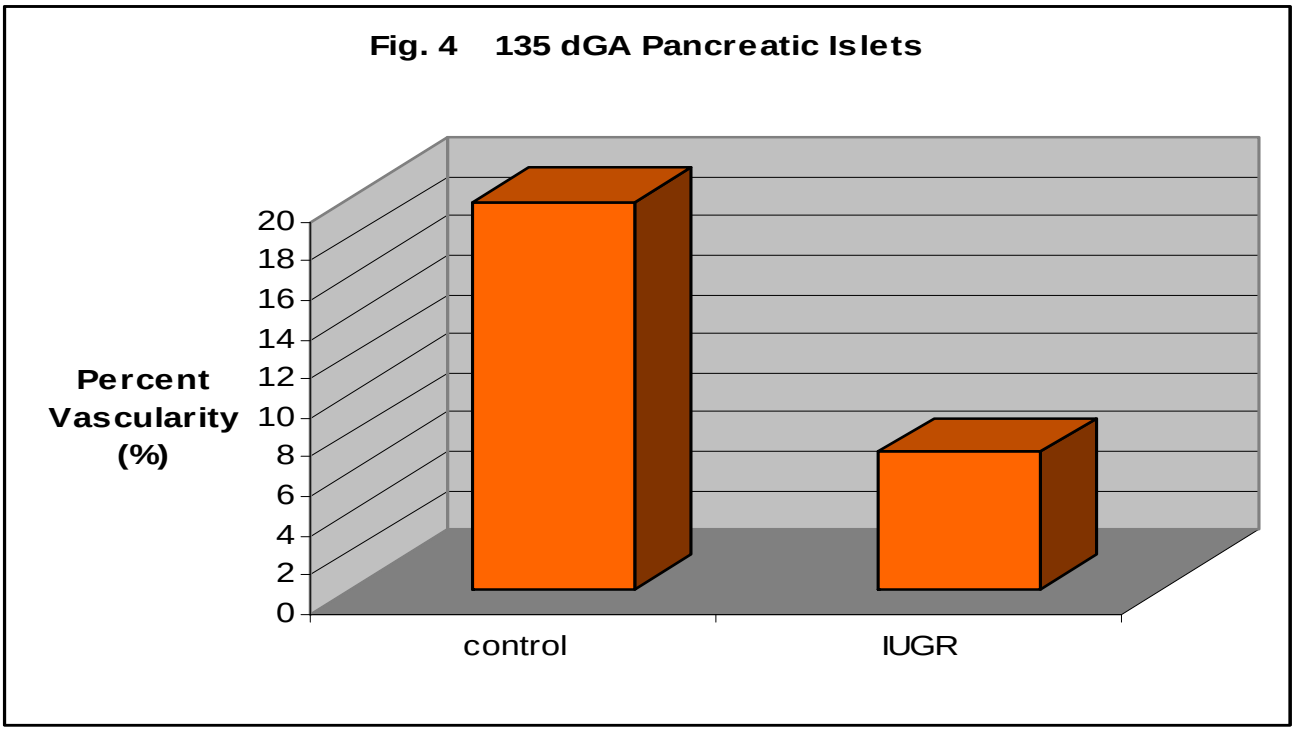


Fig. 3 & 4 represent the percent vascularity of overall pancreas tissue (Fig.3) and pancreatic islets of Langerhans (Fig. 4) in fetal sheep at 90% gestation (135 dGA). Images of the tissue were obtained and the overall area of the image was determined. The area of vasculature was determined by staining the vasculature with GS1 FITC. The percent vascularity was then calculated.

Fig. 5 Lamb Pancreatic Tissue

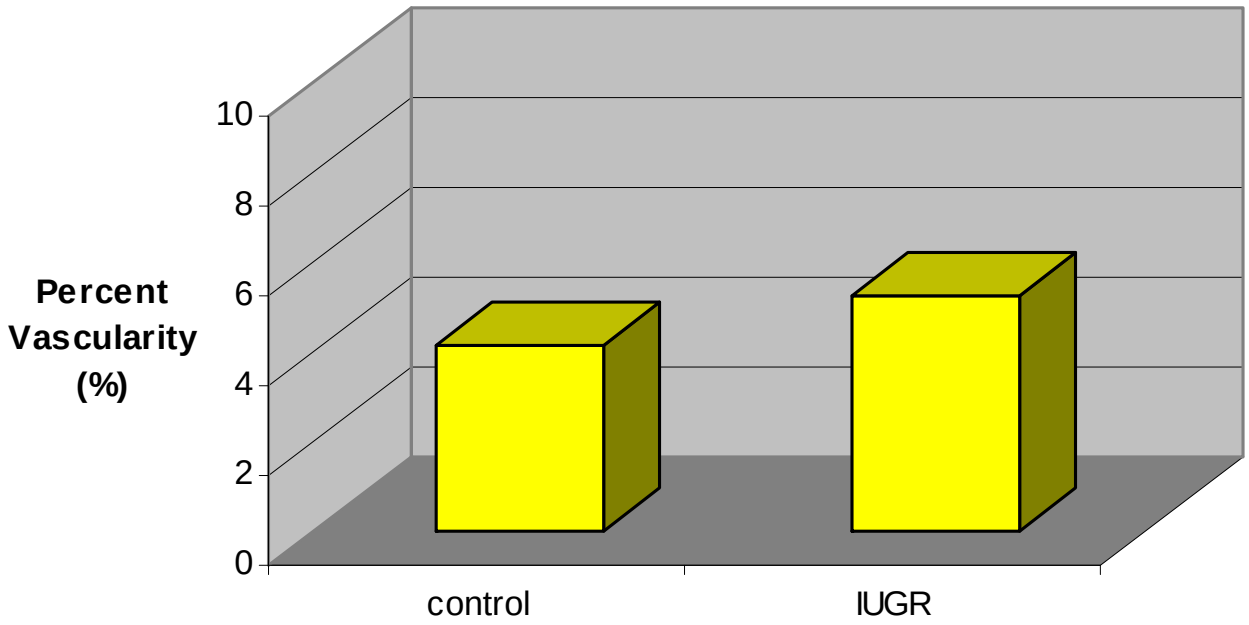


Fig. 6 Lamb Pancreatic Islets

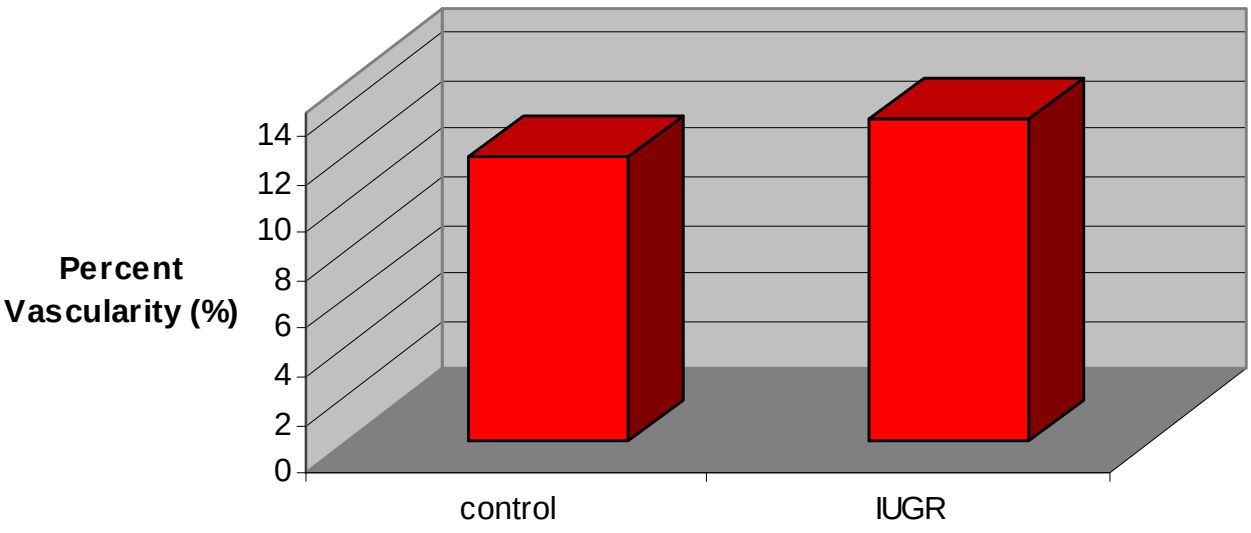


Fig. 5 & 6 represent the percent vascularity of overall pancreas tissue (Fig.5) and pancreatic islets of Langerhans (Fig. 6) in post-natal lambs (3 weeks after birth). Images of the tissue were obtained and the overall area of the image was determined. The area of vasculature was determined by staining the vasculature with GS1 FITC. The percent vascularity was then calculated.

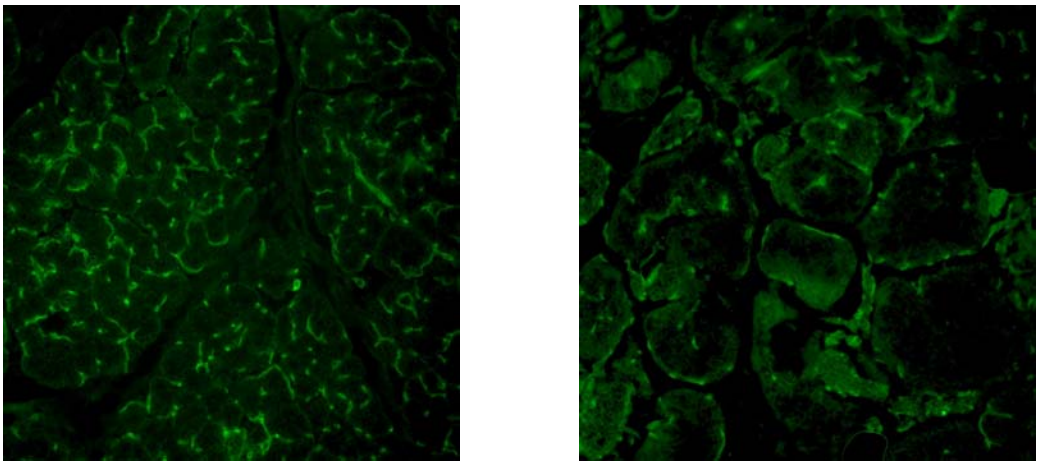


Fig. 7 Images of control (left) and IUGR (right) 135 dGA pancreatic tissue stained with GS1 FITC. The vasculature fluoresces green in each image. The representative control sample shows 7% vascularity and the IUGR sample shows 4% vascularity.

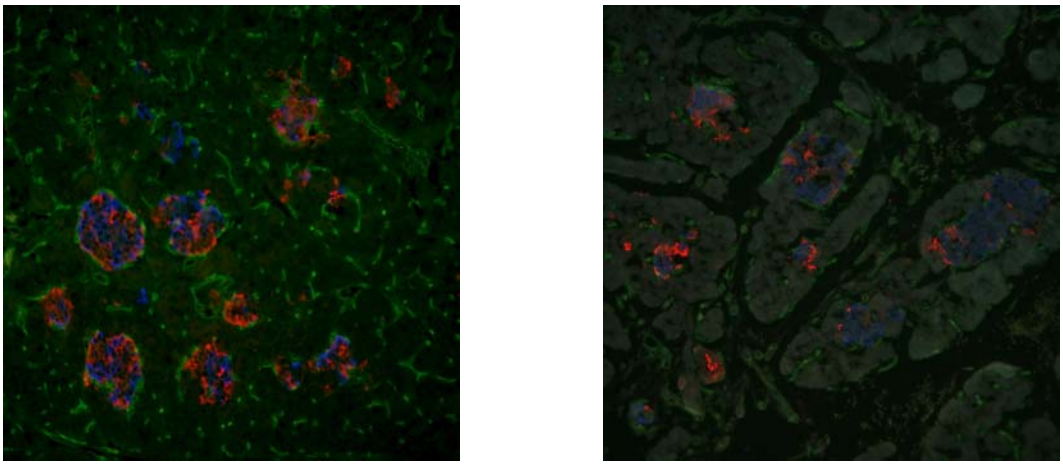


Fig. 8 Representative images of control (left) and IUGR (right) 135dGA tissue containing pancreatic islets of Langerhans stained with AMCA Blue, Texas Red, and GS1 FITC. The vasculature fluoresces green in each image. Each islet was analyzed individually to determine percent vascularity.

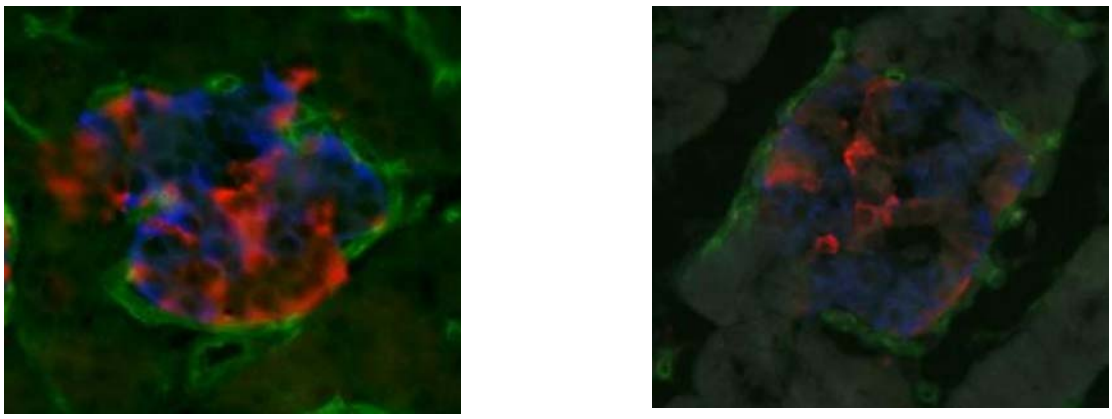


Fig. 9 Representative images of control (left) and IUGR (right) 135dGA pancreatic islets of Langerhans stained with AMCA Blue, Texas Red, and GS1 FITC. The vasculature fluoresces green in each image. The representative control sample shows 20% vascularity and the IUGR sample shows 7% vascularity.