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EFFECTS OF ATORVASTATIN ON THE REGENERATION OF PANCREATIC BETA CELLS AFTER STREPTOZOTOCIN TREATMENT IN THE NEONATAL RODENT

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EFFECTS OF ATORVASTATIN ON THE REGENERATION OF PANCREATIC BETA CELLS AFTER STREPTOZOTOCIN TREATMENT IN THE NEONATAL RODENT

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ABSTRACT

In order to investigate the role of statins in beta (β)-cell regeneration, we used a model of streptozotocin (STZ)-induced β-cell injury, and attempted to affect neovascularization using atorvastatin (AT), in order to aid in β-cell recovery. The recruitment of EPCs, which readily home to sites of injury, would contribute to new blood vessel formation and deliver appropriate signals for β-cell regeneration. We hypothesized that the rate and capacity of the β-cell growth and regenerative processes would increase with AT.

β-cell mass was expanded by postnatal day (PD) 44 and we observed a better glucose tolerance in STZ animals treated with 20 mg/kg AT. This treatment appeared to affect the neogenesis of islets and caused an increase in the number of endothelial cells (ECs). Our findings suggest a novel effect of statins and will help further elucidate the mechanisms of β-cell regeneration.

Keywords: streptozotocin, beta-cell, atorvastatin, endothelial progenitor cells, endothelial cells, regeneration

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ABBREVIATIONS

A.C.N	acetonitrile
AD	antibody diluent
amu	atomic mass units
AT	atorvastatin
ATP	adenosine triphosphate
BrdU	bromodeoxyuridine
BS-1 lectin	*Griffonia (Bandeiraea) Simplicifolia* lectin I
BSA	bovine serum albumin
CAD	coronary artery disease
Cdh5	cadherin 5, type 2, VE-cadherin (vascular epithelium)
CK20	cytokeratin 20
C_{max}	maximum plasma concentration
CMC	carboxymethylcellulose
CO_2	carbon dioxide
cpm	counts per minute
d	day
DAB	diaminobenzedine
dH_20	deionized water
DNA	deoxyribonucleic acid
DP	dorsal pancreatic bud
e	embryonic day
EC	endothelial cell

EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EPC	endothelial progenitor cell
ESI	electrospray ionization
EtOH	ethanols
eV	electron Volts
Flk1	Fetal liver kinase 1
Flt1	FMS-like tyrosine kinase 1
g	gram
GD	gestation day
GH	growth hormone
g/kg	gram per kilogram
GLP-1	glucagon-like peptide-1
Glut2	*glucose transporter 2*
GTT	glucose tolerance test
h	hour
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HGF	hepatocyte growth factor
HIER	heat induced epitope retrieval
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC/MS	high performance liquid chromatography/ mass spectrometry

IGF	insulin-like growth factors
ip	intraperitoneal
ipGTT	intraperitoneal glucose tolerance test
iv	intravenous
kg	kilogram
Kit	kit oncogene
M	molar
MeOH	methanol
mg	milligram
mg/dL	milligrams per decilitre
mg/kg	milligram per kilogram
min	minute
ml	millilitre
ml/mg	millilitre per milligram
ml/min	millilitre per minute
mm	millimetre
mm^2	millimetre squared
mM	millimolar
mRNA	messenger ribonucleic acid
M/Z	mass to change ratio
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide

ng/ml	nanogram per millilitre
Nkx2-2	NK2 transcription factor related, locus 2
Nkx6-1	NK6 transcription factor related, locus 1
NOD/SCID	nonobese diabetic/severe combined immunodeficient
Neurog3	neurogenin3
oGTT	oral glucose tolerance test
Pax4	paired box gene 4
Pax6	paired box gene 6
PBS	Phosphate Buffered Saline
PD	postnatal day
Pdx1	pancreatic and duodenal homeobox 1
PECAM1	Platelet Endothelial Cells Adhesion Molecule 1
PGF	placental growth factor
pM	picomolar
PP	pancreatic polypeptide
Q	quadrupole
RIA	radioimmunoassay
RPM	revolutions per minute
RS	rosuvastatin
RT	room temperature
RTV	Real Time Viewing
SPE	solid phase extraction
STZ	streptozotocin

$t_{1/2}$	apparent half-life
Tek	endothelial-specific receptor tyrosine kinase
t_{max}	time of maximum plasma concentration
TSQ	triple stage quadrupole
TTBS	Tris Buffered Saline-Tween 20®
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor-1
VEGFR-2	vascular endothelial growth factor receptor-2
vol/vol	percent volume in volume
VP	ventral pancreatic bud
Vwf	von Willebrand factor
wk	week
wt/vol	percent weight in volume
°C	degrees celcius
Å	angstroms
α	alpha
β	beta
δ	delta
ε	epsilon
µg/g	microgram per gram
µg/ml	microgram per millilitre
µl	microlitre
µm	micrometre

μm^2	micrometre squared

CHAPTER 1

INTRODUCTION

1.1 Diabetes

The prevalence of diabetes in all its different forms is increasing at epidemic proportions. It is estimated that at least two hundred million individuals currently suffer from the disease (1), and this number is expected to double by 2025 (2).

1.1.1 Diabetes: types, treatments and complications

There are two main types of diabetes mellitus, although new forms of the disease are emerging. Type 1 diabetes, which encompasses less than 10% of the population, is found most often in children and adolescence. It is caused by the autoimmune destruction of the β-cells, which reduces insulin secretion. Survival of these individuals depends on multiple daily insulin injections (3-6). Type 2 diabetes, which is often associated with obesity, is becoming more evident in younger individuals. It is caused by the combination of insulin resistance and inadequate insulin secretion. Individuals with type 2 diabetes are initially managed with lifestyle changes and oral hypoglycaemic agents, with the addition of insulin therapy in the later stages of the disease (2;3;7;8).

Due to the deficient β-cell function, individuals with diabetes are unable to produce or secrete insulin in response to increasing blood glucose. Although insulin regimes can improve blood glucose levels, exogenous insulin administration cannot ensure continuous blood glucose control and prevention of diabetic complications including lower limb ischemia, neuropathy, retinopathy, kidney and cardiovascular diseases (4;9;10).

1.1.2 Islet transplantation

A cure or alternative treatments for diabetes might be achieved by replacing lost β-cells. This was demonstrated in an animal model several decades ago when rats that were

rendered diabetic by the β-cell toxin STZ, were treated by injection of isogeneic islets (11). Currently, islet transplantation represents an attractive approach for the treatment of diabetes, however; there are still many limitations to this procedure. The immunosuppressive drug regimen necessary to protect the islets from a recurrent autoimmune attack and rejection of the graft may with time irreversibly damage kidney function. The process of islet isolation itself, even though it has drastically improved during the past few years, still damages transplantable islets and is not as efficient as originally thought. Consequently, two to three donors are necessary in order to obtain a sufficient β-cell mass for transplantation into a single recipient, and there is limited availability of these cadaveric donors. (12).

The Edmonton protocol was designed using a glucocorticoid-free immunosuppressive protocol with infusion of an adequate mass of freshly prepared islets from two or more pancreases from deceased donors (13). Before the Edmonton protocol in 2000, only 8% of patients that underwent pancreatic islet transplantation were free of insulin therapy at one year (13;14). Their advances in islet isolation and immunosuppression had greatly improved the survival and function of islet allografts but a follow-up study of the Edmonton protocol at multiple centers concluded that only 31% of patients were insulin-independent at two years (15). Although transplantation procedures can restore glucose homeostasis in diabetic individuals, it has been shown that it is only for a limited period of time and insulin independence is not permanent (12).

1.1.3 A novel approach to finding a treatment for diabetes

Due to the fact that islet transplantation is not optimally effective, a considerable effort is being made to identify alternative sources of glucose-responsive, insulin-secreting cells for β-cell replacement, whether it be by generating the β-cells *in vitro* for

transplantation or understanding the mechanisms involved in the expansion or regeneration of the β-cell population *in vivo* (12;16;17). A treatment for diabetes will therefore require effective regeneration or replacement of insulin-producing cells, and understanding the capacity and limitations of these processes is vital.

1.2 Anatomy and physiology of the pancreas

The pancreas serves two major functions: the production of digestive enzymes, which are secreted by exocrine acinar cells into the intestine by a branched ductal network and the regulation of blood glucose homeostasis, which is achieved by the endocrine cells of the islets of Langerhans (3;16). In addition to the glandular components, the pancreas has a rich blood supply. The arterial blood passes through each lobule, first to the islets and then to the adjacent acini (3).

The endocrine cells are mainly grouped in the islets of Langerhans, which are compact spheroid clusters embedded in the exocrine tissue. There are four principle endocrine cell types: the β-cells which secrete insulin and make up the majority of cells in the islet, approximately 50 to 80% depending on the species (18;19), the α-cells which secrete glucagon, the delta (δ)-cells which secrete somatostatin, and the pancreatic polypeptide (PP)-cells which secrete pancreatic polypeptide (3). A new endocrine cell type, epsilon (ε)-cells, which produce ghrelin, have been recently identified (20;21). In rodents, there is a defined compartmentalization of cells within the islets; the β-cells lie in the centre (core) and the other cell types at the periphery (mantle), while in humans this grouping is less apparent (3). The proportion of endocrine cells is about 4% of the total cells in the adult rat pancreas (22).

1.3 Pancreatic development and growth

The growth and development of the pancreas has been studied extensively since the nineteenth century (3). Additionally, there is increasing evidence that the stages of pancreatic development are recapitulated during regeneration. In order to identify the development and differentiation processes of the endocrine pancreas, we need to understand the genetic background and the factors that control the growth of the pancreas.

1.3.1 Embryonic and foetal development

During embryonic development, both endocrine and exocrine cells develop from common stem cells within the early gut endoderm (23). The pancreas originates early in development at embryonic day (e)8.5 to e9.5 in the mouse, by two buds of cells (ventral and dorsal) developing from the prepatterned endodermal epithelium (primitive gut endoderm) (24;25). By e10.5, the partially differentiated epithelium of the two buds form highly branched structures (26;27), which develop into a ductal tree that results in the formation of two separate primitive pancreas organs, consisting predominantly of undifferentiated ductal epithelium by e12.5. (Figure 1.1; first transition).

Between e13 and e14, the dorsal and ventral pancreata rotate and fuse into a single definitive pancreas. Vascularization occurs from about e14 in the mouse (3). On e14.5, the exocrine pancreas differentiates from the ductal epithelium and by e15.5, acini are clearly distinguishable from the ducts. In the embryo, the endocrine cells arise at the very beginning of bud development (e9.5) from stem/progenitor cells located within the early gut endoderm, but until e14 are single cells or small clusters within the ductal epithelium (25;28;29), after which they undergo extensive proliferation (Figure 1.1; second transition).

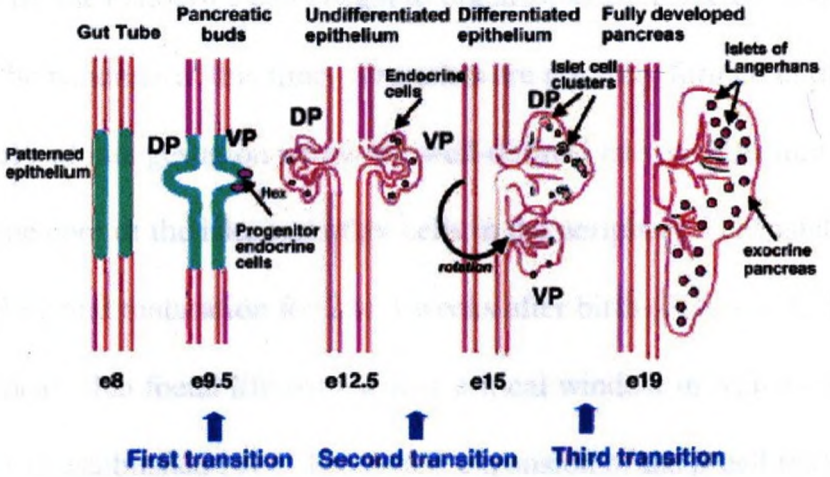

Figure 1.1. Schematic diagram of pancreatic development in the mouse. On e8, prepatterned endodermal epithelium of the foregut forms dorsal (DP) and ventral (VP) pancreatic buds by e9.5, which then develop into branching ducts and undifferentiated epithelium (e12.5; first transition). Single exocrine cells are interspersed among the undifferentiated epithelium. The buds begin to differentiate into endocrine and exocrine cellular lineages by e14 and proliferate and expand extensively (second transition). By e15, the dorsal and ventral pancreases rotate, fuse, and form a nearly fully developed pancreas by e19, containing the endocrine cells organized into isolated clusters that condense into the islets of Langerhans (third transition). The third transition, consisting of maturation of the endocrine cells, continues for 2 to 3 weeks after birth. The approximate embryonic age (e) in days is designated for each stage of development (Modified from 30).

On e16, the endocrine cells begin to organize as islet-like clusters and makeup about 10% of the pancreas at this time. The islets are not fully formed until shortly before birth on e18-e19 of late gestation where the well-defined islet architecture is observed, with β-cells in the core of the islet and other cells in the periphery. The islets undergo additional remodelling and maturation for 2 to 3 weeks after birth (Figure 1.1; third transition).

It appears that foetal life represents a critical window in which the appropriate number of β-cells is established (31). The fastest expansion of the β-cell mass occurs in late foetal gestation, with an approximate doubling of the β-cell population each day starting from the 16th day post conception in rats (32). In the rat foetus where the β-cell number increases at a rate of approximately 100% per day, β-cell division can not account for more than 10 to 20% of the total growth (33). The remaining 80% or more has been attributed to the process of neogenesis, which is the formation of new β-cells from rapidly proliferating, undifferentiated precursor cells (31). In rodents, mature islets, consisting of an outer layer of α-cells and PP cells and an inner mass of β-cells and δ-cells, are formed a few days before birth (34). The islets double in volume just before term due to β-cell replication, as well as recruitment and maturation of undifferentiated β-cell precursors (28;35).

1.3.2 Transcriptional control of development

In order to create mature endocrine cells an ordered and coordinated expression of transcription factors are needed (17;26). The transcription factor, pancreatic and duodenal homeobox 1 (Pdx1) functions at multiple stages of pancreas development and is a master regulator of both pancreatic development and the differentiation of progenitor cells into the β-cell phenotype (36;37). Pdx1 expression is important during early stages of development (38) as it plays a vital role in the initial morphological development of the

pancreas and initiates endocrine lineage commitment for cells within the pancreatic ducts. During foetal development, Pdx1 becomes restricted to differentiating β, δ and PP cells, and then is lost from putative β-cells, and finally becomes restricted to mature β-cells where in the adult pancreas, it controls insulin and glucose transporter 2 (Glut2) expression (17;26;39;40).

In the generation of the pancreas, the transcription factor neurogenin3 (Neurog3), is required for the development of all endocrine cell lineages (41), and has been designated a marker of islet precursor cells (42). Expression of neurog3 starts at e9-9.5, peaks at e15.5 during the major wave of endocrine cell neogenesis, and is greatly diminished at birth (26). NK2 transcription factor related, locus 2 (Nkx2-2) is a member of the NK class of homeodomain proteins, and is necessary for the terminal differentiation of pancreatic β-cells into functional insulin-producing cells, and Nkx6-1 is involved in the pathway to differentiate β-cells. Paired box gene 4 (Pax4), a paired domain homeobox gene, functions at a crucial stage in the differentiation pathway, at a point where precursor cells may possess the ability to select different terminal fates. It also plays a role in the differentiation of the mature β-cell and by birth, its expression is restricted to β-cells. Paired box gene 6 (Pax6) is important for islet cell development and expressed in all of the mature endocrine cells. Differentiation of the lineages leading to β-cells and α-cells is determined by the differential expression of Pax4 or Pax6 (17;26;30;43).

1.3.3 Postnatal development

Four basic physiological processes determine β-cell mass: β-cell replication, β-cell neogenesis, changes in size of individual β-cells, and β-cell apoptosis (44;45). In the foetus, there is a rapid neogenesis of islets and this continues throughout neonatal life at a reduced rate (32;35;46), and ceases shortly after weaning (29;35;47). The endocrine

pancreas of the neonatal rat undergoes substantial remodelling during the first two weeks of postnatal life. The incidence of apoptotic cells within the islets increased three fold at PD 14. However, the β-cell mass was not altered appreciably at the time of neonatal apoptosis because a new population of β-cells compensated for the loss. A transient wave of apoptosis facilitates the loss of foetal β-cells and they are replaced with newly derived adult β-cells, which have a more mature phenotype of acute, glucose-sensitive insulin release suited to metabolic control in later adult life (48-51). Some evidence suggests that a similar wave of apoptosis occurs in the human foetus during the third trimester (52). It may be during this period that important changes take place at the level of β-cell maturation.

This wave of cell death between PD 2 and PD 18 is followed by increased neogenesis of islets shortly before weaning (50). Increased numbers of insulin positive cells are seen near the ductal epithelium at PD 12, suggesting the generation of new islets to maintain the β-cell mass. Post-weaning, the rates of β-cell replication, neogenesis and apoptosis decline and by the time the adult pancreatic β-cells mature, they replicate at a very slow rate, about 3% each day (8;50;53;54). The proportion of islet cells in the rat decreases, reaching a plateau of about 4% of total pancreatic cells by three months of age (55). Histologically, dividing β-cells can be found in the adult pancreas, but they are very scarce. The pancreas changes from a very plastic compartment to one with a limited capacity for renewal over the first three months of postnatal life. As a result, the capacity for renewal of the pancreas decreases as a function of time (50).

1.4 Pancreatic regeneration

It has been shown by numerous investigators that the pancreas is an organ that maintains its capacity of regeneration, either by partial or total ablation by surgery or by a

chemical insult (56-58). It is known that the β-cell mass is dynamic, and that holds a potential for targeted islet β-cell regeneration (59). Many potential mechanisms have been proposed including replication of pre-existing β-cells, emergence of new β-cells from precursor cells in the pancreatic ductal epithelium (neogenesis), transdifferentiation of exocrine (acinar cells) into endocrine β-cells or differentiation of pancreatic stem/progenitor cells (Figure 1.2) (59-61). The relative contributions of these processes in maintaining and expanding β-cell mass has not been well-defined and varies between species (62).

1.4.1 β-cell replication

β-cell replication or proliferation is the formation of β-cells from mitotic division of pre-existing mature β-cells (50). β-cells are not a highly proliferative cell type, but evidence that they can replicate has existed for years. This has been confirmed by a variety of methods including incorporation of nucleotide analogues such as bromodeoxyuridine (BrdU) (63), as well as the expression of markers of cell cycle entry such as Ki67 (64).

β-cell replication was found to be significantly higher during late gestation and in the neonatal period than following weaning, with little change of the replication rate of 2 to 3% new cells per day occurring beyond PD 30 to PD 40 in the rodent (50). While data in humans are limited, it appears that human β-cells replicate at a much lower rate than in the rodent (65). β-cell replication in the adult human pancreas was rare at a frequency of 0.001% (62;64). However, evidence has shown that mature murine β-cells retain their proliferative capacity and are the major source of new β-cells in the adult (66). Butler et al. (67) have shown that β-cell mass expansion occurs primarily via proliferation in the mouse while in human patients, β-cell replication is unchanged (62). Although, in one

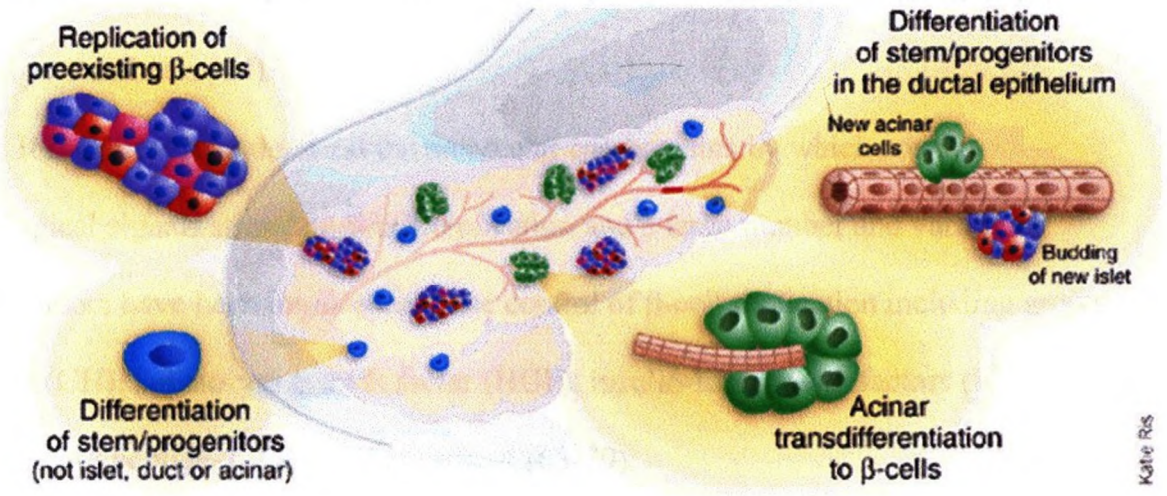

Figure 1.2. The pancreas as a source of new β-cells. The pancreas itself is likely to be the main source of new insulin-producing β-cells and of cells that can regenerate the acini and ducts. Several processes have been proposed: replication of pre-existing β-cells; differentiation of progenitors within the ductal epithelium; transdifferentiation of acinar cells; and differentiation of pancreatic stem/progenitor cells that are not of β-cell, duct or acinar origin (60).

case, a significant increase in β-cell replication was noted in an 89-year old with recent onset type 1 diabetes (64).

It is critical to understand the molecular mechanisms by which normal physiological signals stimulate β-cell replication and a large number and variety of growth factors have been implicated in the control of β-cell replication including growth hormone (GH), hepatocyte growth factor (HGF), insulin-like growth factors (IGF), and glucagon-like peptide-1 (GLP-1)/ exendin-4 (68-70).

1.4.2 β-cell neogenesis

Evidence for β-cell neogenesis comes primarily from morphological studies that have induced total or partial damage to the pancreas. Morphologically, neogenesis is defined as endocrine cells budding from pancreatic ducts and/or clusters of β-cells scattered within the exocrine pancreas (44;71). The origin of the cells that contribute to neogenesis is unknown and endocrine progenitor cells have not been precisely identified because of the lack of specific cell markers (72). Neogenesis could be a result of differentiation of stem cells that reside in the pancreatic islets, ducts, acini, or extra-pancreatic cells could also be involved (44;72;73). Islet neogenesis has been reported to occur in several animal models, including after β-cell destruction with toxins in neonatal rodents (74), after cellophane wrapping of the pancreas in adults (75), and after partial pancreatectomy (76). In these animal models, subsequent to the stimulus, a common finding is that cells expressing insulin appear in locations such as ducts.

Islet neogenesis, while clearly the main pathway of β-cell increase during early to mid-gestation, still occurs in the normal postnatal animal; with a second wave of neogenesis at weaning and a slower rate during adult life (50;53;77). Neogenesis can be enhanced experimentally (76;78), although it is difficult to quantify the amount of

neogenesis as there are neither specific markers of new β-cells nor time denominators for the process, and the signalling response for stimulating neogenesis is still unknown (29). Recent studies by Brand et al. (79) and Rosenberg et al. (80) indicate that in the STZ rat model, islet neogenesis is a significant contributor to β-cell mass expansion. In adult humans, neogenesis appears to be the most significant mechanism in increasing β-cell mass and failure of this process has been blamed for lack of β-cell mass compensation in humans with type 2 diabetes (62).

The theory that neogenesis mimics embryonic development has been supported and much evidence has emerged to suggest that endocrine progenitor cells originate from the pancreatic ducts which was clearly demonstrated in pancreatic injury models in rodents (81). The specific cascade of transcription factor expression that characterizes embryonic growth seems to be involved in neogenesis from ductal progenitor cells, thus recapitulating ontogenic development (26). Zhang and Sarvetnick provide evidence that neogenesis is the same process in the embryo and the adult. They suggested that the adult pancreas had smaller numbers of less active stem cells as a result of a different microenvironment which consisted of mature islets (72).

1.4.3 β-cell transdifferentiation

Transdifferentiation has been described as the phenotypic switch from one differentiated state to another (31). It is not clear whether this mechanism of regeneration may be operational in mammals, although there has been some evidence of this process. In the pancreas it is known that severe tissue injury leads to acinoductal transdifferentiation where acinar cells switch to a duct like phenotype and subsequent islet neogenesis occurs (82). Desai et al. (83) have recently tested the potential contribution of acinar cells to new β-cells formed during pancreatic regeneration and

found that the acinar cells do not give rise to new β-cells during regeneration. This was contrary to evidence observed in previous *in vivo* and *in vitro* studies which found that with the appropriate signals, the acinar cells were able to differentiate into insulin-producing cells (84;85), although the significance of this process in humans is unknown (73).

1.4.4 Differentiation from stem/progenitor cells to β-cells

Adult stem cells exist in most tissues within the body, where they contribute to tissue homeostasis and regeneration. It has been suggested that adult stem cells exist within the pancreas as well, but their identity and location have yet to be elucidated (6;86). Several groups have generated pancreatic β-cells *in vitro* from pancreatic ducts, islets, exocrine cells and non-pancreatic cells, but the extent of differentiation has been extremely low (87-89). Recent work by Seaburg et al. (90) claims the identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages, while lineage tracing studies by Dor et al. (66) suggests an alternative view, that adult pancreatic stem cells do not contribute to new β-cells and that organ maintenance is provided only by replication of pre-existing differentiated β-cells.

Bone marrow is an important source of stem cells (91). Ianus at al. (92) reported that transplanted bone marrow cells contribute substantially to adult β-cells. This study, which did not include diabetic animals, found that under steady state conditions 1.7 to 3% of all β-cells in the mice were of donor origin 4 to 6 weeks after bone marrow transplantation (92). This added pancreatic β-cells to the list of differentiated cells that could be potentially derived from the bone marrow stem cells. However, some of the original studies could not be confirmed by other laboratories (93), so whether β-cells can be derived from bone marrow remains to be determined.

Hess et al. (94) induced a diabetes-like condition in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice by injecting them with STZ. Mice were then intravenously treated with bone marrow cells after sublethal irradiation. Compared to the controls, the mice that received viable bone marrow cells showed lower blood glucose levels and increased β-cell numbers in the pancreas. Progeny of the injected bone marrow cells were found in the pancreas, but only rarely did these cells stain positive for insulin. Instead many of them became vascular ECs, increasing the overall vascularity of the pancreas (94).

Furthermore, in a study by Lechner et al. (95), no significant *in vivo* differentiation of bone marrow into pancreatic islet β-cells was found in adult mice, neither under steady state conditions nor after tissue injury through partial pancreatectomy or STZ administration. The concept put forward by Hess et al. (94) does not rely on transdifferentiation of bone marrow stem cells. The proposed mechanism stays within the traditional framework of germ layer specification, in which bone marrow stem cells differentiate to vascular EC. Signalling from the vascular cells has recently been shown to be critically important in early pancreas development (25).

Rather than serving as progenitors, bone marrow cells could facilitate new β-cells formation. In mice with STZ-induced islet injury and resultant diabetes, homing of transplanted marrow cells to the pancreas was associated with normalization of glycemia and increased islet cell mass (94;96). Many of these bone marrow cells were found to have an endothelial phenotype but there was very little or no evidence of them turning into β-cells.

1.5 Experimental models of regeneration

Although the embryonic origins and development of the islets of Langerhans have been extensively studied (17;43), it is not clear whether regeneration postnatally involves the same pathways or whether β-cells derive from different precursor populations. Regeneration of pancreatic β-cell mass following toxic, surgical or autoimmune-mediated destruction is possible in the young rodent, but the extent of recovery decreases with age and is limited in adult life. The capacity of postnatal β-cells to grow or to regenerate seems to be possible as several experimental models have shown that under certain conditions and at certain stages of development, the growth of β-cells can be reactivated (31). Regeneration of the endocrine pancreas has been studied in many experimental models that differ in the extent and selectivity of the tissue injury that is inflicted (3;Reviewed in 81).

1.5.1 Pancreatic ductal ligation

It has been known for several years that pancreatic ductal ligation can trigger islet neogenesis. Partial ligation of the main duct prevents the outflow of pancreatic secretions and this leads to a rapid destruction of the acinar tissue and a slower destruction of ducts and islets (97). The part of the pancreas that lies downstream to the ligation continues to function normally. It was shown in a ligated rat pancreas that the β-cell population nearly doubles within the first week after duct ligation. The observed increase in β-cell number was 80% in only 7 days (98). β-cell replication was only slightly elevated which suggests that the increased β-cell number results from neogenesis, as was evident when cytokeratin 20 (CK20)-positive ductal cells were identified that co-expressed insulin or the β-cell marker, Glut2. These results suggested that neogenesis of β-cells from ductal cells, rather

than replication from pre-existing β-cells, was the predominant method of regeneration (98).

1.5.2 Cellophane wrapping

Islet cell neogenesis can also be induced in this model of partial pancreatic duct obstruction in hamsters (75;78). In this model, evidence is provided for a continuous development from duct to islet cells. The continuous inflammation leads to some new islet formation by budding from ductules. A way to provoke this was to wrap the head of the hamster pancreas with cellophane tape which lead to local inflammation and fibrosis (78). Fourteen days after the head of the pancreas had been wrapped in cellophane; cells migrated from the epithelium of small intralobular ductules and began to form new islet structures. As well, there was some budding of islets from hyperplasic ductules. After 8 weeks, there was a two-fold increase in islet cell mass. This ductal cell proliferation lead to a cell outgrowth after 21 days, forming new, small islets containing either glucagon, insulin, or both (75;78).

1.5.3 Partial pancreatectomy

The partial pancreatectomy rat model, where 90% of the pancreas is removed surgically, is another model for studying the mechanisms of growth and regeneration, as part of the rat pancreas can be removed without serious effects on the animal's growth rate (99;100). This 90% resection of the pancreas in the neonatal rat induced regeneration of both exocrine and endocrine tissue (101). By 8 days after surgery, the remaining 10% of the pancreas had regenerated to 27% of the normal pancreas weight and 45% of the islet cell mass (76). Following the pancreatectomy, there was only limited regenerative growth and there was never complete restoration of the original pancreatic volume (31). Furthermore, the regenerative response was proportional to the amount of pancreas

removed (102). New β-cells were derived by both neogenesis from proliferating ducts (76), and cell replication within the remaining islets (103). Contrary to earlier beliefs, cell lineage marking of β-cells in mice followed by a partial pancreatectomy showed that the new β-cells in the remaining organ are derived almost exclusively from existing β-cells in adult life (66).

1.5.4 Pancreatic alloxan perfusion

Alloxan has been used as a model to induce diabetes because it specifically destroys β-cells by rapid necrosis (104). Alloxan is a generator of oxygen free radicals causing extensive deoxyribonucleic acid (DNA) damage (105). Waguri et al. (106) established a model of transient diabetes for 48 weeks in mice by perfusing the body and tail of the pancreas with alloxan after clamping the superior mesenteric artery. In these animals, all β-cells were destroyed within 5 days and in this model, both processes; proliferation and differentiation, took place in the same pancreas. In the β-cell-depleted portion, neogenesis of β-cells from extra-islet precursors located within the ductal epithelium was mainly observed while in the control non-alloxan perfused portion of the pancreas, β-cell regeneration was based mainly on proliferation from pre-existing β-cells located in the islets (106).

1.5.5 Pancreatic STZ treatment in the neonatal rodent

STZ causes rapid necrosis of β-cells, and is preferred over alloxan because it has fewer side effects (104). STZ is a 2-deoxy-2-(3-(methyl-3-nitrosourea)-D-glycopyranose) molecule that produces a selective toxic effect on β-cells (105;107). It is believed that the basis of the selective effect of STZ on β-cells is its glucose moiety, which reacts with the specific glucose sensing mechanism (108;109), and is thought to be due to a better uptake by the β-cells via the glucose transporter, Glut2 (105). The exact

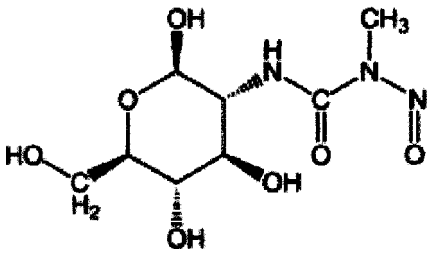

Figure 1.3. The structure of streptozotocin. STZ is a 2-deoxy-2-(3-(methyl-3-nitrosourea)-D-glycopyranose) molecule that produces a selective toxic effect on β-cells due to its glucose moiety, which allows it to be taken up by the glucose transporter, Glut2 (Modified from110).

mechanism of STZ toxicity is not known but its primary effect on β-cells is DNA damage including alkylation and DNA strand breaks by free radicals generated when the compound decomposes inside the cell (31;111-114). The DNA strand breaks in the β-cells deplete the nicotinamide adenine dinucleotide (NAD) levels in the cell and further reduce adenosine triphosphate (ATP) content. STZ actions in β-cells are accompanied by characteristic alterations in blood glucose and insulin concentrations. STZ impairs glucose oxidation (115) and decreases insulin biosynthesis and secretion (114;116;117) which produces long-lasting impairment and death (108;118).

β-cell growth after injury has been studied extensively in rats during the neonatal period and it has been shown to result in substantial β-cell replacement, although the animals are predisposed to glucose intolerance later in life (54;56;74;119-122). Given on the first day of life, STZ induces subtotal β-cell damage with associated hyperglycaemia, which is followed by rapid β-cell regeneration restoring normoglycaemia (46;120). From PD 4 onward, signs of regeneration were apparent, as numerous insulin-positive cells were found throughout the acinar tissue and within the ductal epithelium (57). Although β-cell regeneration increased β-cell number to almost 50% of the normal values by 6 weeks of age, β-cell function was diminished, resulting in glucose intolerance (56;57). In this STZ model, the proliferative activity of β-cells was increased, but insufficient to regenerate a functional β-cell mass (56).

Near-complete removal of β-cells leads to partial replacement through the appearance of newly formed islets, whereas subtotal destruction is followed by renewal from within the islets (56;119;120;123;124). Wang et al. (54) showed that the extent of β-cell regeneration depends on the timing of β-cell damage. There is the capacity of β-

cell regeneration in the neonatal rodent pancreas although it decreases quickly during the first postnatal week (120) and it is lacking or very limited in the adult rodents (54).

The period of natural developmental plasticity of the β-cells allows for studying the mechanisms of growth and regeneration of the pancreas, as well as, possible identification of the mechanisms needed for β-cell replacement after artificially inducing regeneration by destruction of the β-cells with STZ (120;122). Previously our laboratory has shown that induced damage to the β-cells of neonatal rodents by administration of STZ resulted in β-cell regeneration (122). We showed that a 70 mg/kg dose of STZ administered to neonatal rats at PD 4, resulted in destruction of approximately 60% of the β-cells in the islets within 72 hours and caused temporary hyperglycaemia that returned to normal physiological range by PD 40 (122). The changes seen in the damaged pancreas are comparable to the remodelling seen during normal islet development in rodents between PD 7 and PD 21. The β-cell destruction by STZ was rapidly reversed with β-cell mass having returned to 70% of control values by PD 40. The growth was due to both a re-population from within islets and from neogenesis at the pancreatic ducts (122). Unfortunately, the mechanisms that initiate β-cell regeneration are still poorly understood.

1.6 Role of the vasculature during pancreatic development

The pancreas and the vasculature show a strong interaction starting early in development and maintain this interaction throughout islet formation. Vascular and pancreatic development coincide during embryogenesis (125;126). Lammert et al. (125) established that the blood vessel endothelium induces endocrine pancreatic differentiation and provides metabolic sustenance for endocrine cell development and growth, as well as the inductive signals for organ development (Figure 1.3). Vascular EC contact and

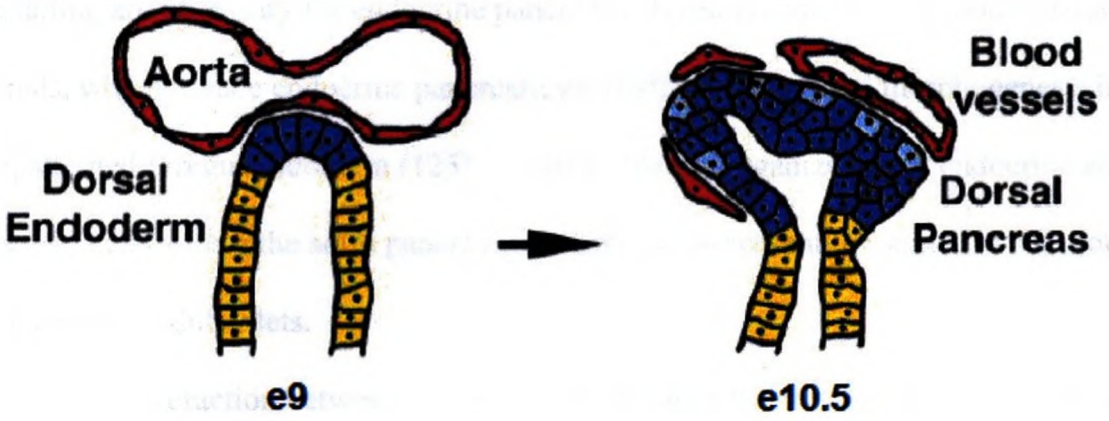

Figure 1.4. Blood vessel and organ interactions during pancreatic development at embryonic day (e)9 and e10.5. The aorta endothelium is adjacent to the developing dorsal pancreatic endoderm. The endothelium induces budding and endocrine development. Blood vessels residing in the pancreatic mesenchyme continue to interact with the adjacent pancreatic epithelium throughout development. (*red* vascular endothelium, *blue* pancreatic endoderm, *light blue* endocrine cells, *yellow* nonpancreatic gut endoderm) (126).

signalling are necessary for endocrine pancreatic development. ECs harbour instructive signals, which induce endocrine pancreatic cell differentiation and morphogenesis in the prepatterned foregut endoderm (125). Considering the organization of endocrine and vascular cells within the adult pancreas, it seems plausible that the same signals would play a role in adult islets.

The interaction between the blood vessels and endocrine cells continues when mature hormone-secreting islet cells are formed. The pancreatic islets of Langerhans are well vascularized throughout life. This is critical for proper metabolic sensing and homeostatic regulation mediated by these cells (125). In rodents, each islet of Langerhans receives its blood supply from one to five afferent arterioles that branch into a glomerular-like network of microvessels and form a local intra-islet portal system through which the blood flows from the central core of β-cells to the mantle of the islet (127). Endocrine microvessels are thinner walled than exocrine capillaries which are important for glucose sensing and insulin secretion (128).

1.7 Characteristics of EPCs

Circulating EPCs were identified and implicated in postnatal angiogenesis (129-131). The bone marrow appears to be a reservoir for EPCs (132), but they are also present in the systemic circulation, and are augmented in response to certain cytokines and localize to, as well as incorporate into, sites of neovascularization. Asahara et al. (129) first described EPCs as bone marrow-derived circulating angioblast precursor cells. They are detectable in the blood and bone marrow throughout life, although aging is correlated with decreasing numbers of circulating EPCs (133;134). They have the capacity to proliferate, migrate, and differentiate into endothelial lineage cells, but have not yet acquired the characteristics of mature ECs (135). Urbich and Dimmeler (136) define an

EPC as non-EC that has the ability to multiply, proliferate, and is capable of differentiating into EC. Recent studies provide evidence that progenitor cells can change their phenotype by adapting to the local tissue microenvironment (137). Circulating bone marrow-derived EPCs have been shown to localize to sites of endothelial disruption and become incorporated into growing endothelium (138).

1.7.1 EPC markers

EPCs can be isolated from peripheral, umbilical cord and bone marrow blood (139). Currently, AC133 or CD133, expressed on haematopoietic stem cells, but not on mature ECs, represents the best selective marker for identifying circulating EPCs (131;140), although there is no exclusive EPC marker. Even though the exact phenotype of EPCs has not be definitively established yet, there is a general agreement for the use of at least one additional marker reflecting endothelial commitment; the most used being vascular endothelial growth factor-receptor 2 (VEGFR-2)/Flk1, as well as expression of other endothelial markers such as von Willebrand factor (Vwf), cadherin 5, type 2, VE-cadherin (vascular epithelium) (Cdh5), and Platelet-Endothelial Cells Adhesion Molecule 1 (PECAM1), kit oncogene (Kit), and vascular endothelial growth factor receptor-1 (VEGFR-1) (129;136;141-144). Isolated EPCs expressed several markers in common with ECs such as CD34, PECAM1, VEGFR-2 and endothelial-specific receptor tyrosine kinase (Tek) (145).

1.7.2 EPCs and blood vessel formation

Prior to the discovery of EPCs, new blood vessel formation was believed to occur by the proliferation of existing ECs. These findings have overturned the previous dogma that vasculogenesis can only occur during embryogenesis. In fact, vasculogenesis and angiogenesis may occur simultaneously in postnatal life because EPCs are able to

differentiate when needed into vascular endothelium through a mechanism recapitulating embryonic vasculogenesis (135).

Postnatal neovascularization was originally recognized to be constituted by the mechanism of angiogenesis, a remodelling process characterized by the sprouting of new blood vessels from pre-existing ones, formed by proliferation and migration of pre-existing ECs (146). However, the isolation of EPCs resulted in the addition of a new mechanism, vasculogenesis, which is *de novo* vessel formation by the differentiation, migration, and/or proliferation of bone marrow-derived EPCs (147). Vasculogenesis was originally thought to occur only during early embryonic development. There is strong evidence suggesting that vasculogenesis does make a significant contribution to postnatal neovascularization. Bone marrow EPCs have been shown to contribute to neovascularization in several models including retinal, myocardial, and hind limb ischemia (129;148-150). A subsequent study demonstrated that a CD34/Kit positive-bone marrow cell fraction was capable of integration into vascular endothelium in the pancreas of the diabetic animal (96).

EPC recruitment to sites of neoangiogenesis is triggered by the increased availability of angiogenic growth factors or chemokines, such as VEGF (151). Tissue injury and ischaemia are potent stimuli for neovascularization. Vascular trauma may induce the release of cytokines that promote rapid mobilization of circulating EPCs to the peripheral circulation (152). Once at the site of neovasculazation, the EPCs may recruit additional EPCs by releasing growth factors such as VEGF or HGF (153). Mobilization or transplantation of EPCs can enhance neovascularization and is associated with tissue regeneration and functional improvement after injury (148;154;155).

1.7.3 Vascular endothelial growth factor (VEGF)

Among factors that control the formation of blood vessels is VEGF, which is a strong mitogenic factor for ECs in various *in vivo* and *in vitro* systems (156;157), and has been shown to increase the permeability of microvessels (158;159). VEGFA, a key regulator of blood vessel growth, belongs to a gene family that includes placental growth factor (PGF), VEGFB, VEGFC, VEGFD. The human VEGFA has four different isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (160). VEGFA binds with high affinity to two tyrosine kinase receptors expressed mainly by ECs: Flk1/VEGFR-2 (161;162) and Flt1/VEGFR-1 (163). VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF (160). Islet β-cells express significant levels of VEGFA (25;125) which induce proliferation, migration and differentiation of ECs, as well as the growth of blood vessels (126). The β-cells express high levels of VEGFA in order to attract VEGFR-2-expressing ECs, which form a vascular network within the islets (125;164). This dense vascular network is required for proper endocrine function and islet size (125).

1.8 Statins enhance EPCs

Recently several studies demonstrated a novel function for statins, showing that they contribute to postnatal neovascularization by increasing the mobilization of bone marrow-derived EPCs (142;143;165). Vasa et al. (143) demonstrated that the positive effects of statins on EPCs include increasing the number of circulating EPCs, reducing senescence, and enhancing proliferation and differentiation.

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are known as cholesterol-lowering drugs that block the rate limiting step of cholesterol biosynthesis (142;166). In addition to this, statins possess cholesterol-

independent, pleiotropic effects which include the promotion of angiogenesis, and re-endothelialization by inducing the mobilization and differentiation of EPCs *in vitro* and *in vivo*. This contributes to neovascularization and therefore has a direct influence on ECs (138). The pro-angiogenic effects of statins have been documented to occur at low-dose levels during *in vitro* or *in vivo* experiments corresponding to physiological levels achieved in clinical practise, which would be equivalent to a patient dosage of 40 mg/day (138). Some authors argue that achievement of pleiotropic effects in mice may require a dose of up to 40 mg/kg/day (167).

Statins have been shown to increase the number of EPCs *in vitro*, in animal models and in patients with coronary artery disease (CAD) (142;143;165). Studies have demonstrated that EPCs from healthy volunteers and CAD patients can transdifferentiate *in vitro* into functionally active cardiomyocytes when co-cultured with cardiomyocytes requiring either cell-to-cell contact or cellular fusion to mediate EPC transdifferentiation (168-170). Statin therapy in patients with CAD improved the impaired EPC differentiation into cardiomyogenic cells (166). Thus, the therapeutic use of autologous EPCs may stimulate cardiomyocyte regeneration in patients with ischemic heart disease, thereby potentially contributing to organ-specific regeneration (166).

1.8.1 Experimental approach for selection of statin

AT exhibits a number of characteristics that are different from other members of the statin drug family including longer plasma half-life and active metabolites that have equivalent pharmacological activity to that of the parent drug. These characteristics are postulated to be responsible for a more prolonged effect and greater efficacy of AT (171).

The majority of statins including, lovastatin, fluvastatin, and simvastatin, are developmentally toxic in rats (172;173), except for AT which has been reported to have

no teratogenic effects (174;175). No adverse effects on fertility or reproduction were observed in females rats given doses up to 225 mg/kg/day (174;176). Developmental toxicity was observed with the administration of 225 mg/kg of AT, and reduced offspring body weight and behavioural changes also occurred at 100 mg/kg, a dose which did not result in adverse effects on the maternal animal (174;176). These doses are 100 to 140 times the recommended human dose. A 20 mg/kg/day dose had no adverse effects on the offspring (176). As well, a single dose of 10mg/kg AT given to female rats on gestation day (GD) 19 or PD 13 provided evidence of placental transfer and excretion into the milk (176), an important characteristic for the continued transfer of AT during neonatal life.

1.8.2 Pharmacokinetics of AT

In rodents, AT undergoes metabolism similar to that in humans (177). AT is rapidly absorbed after oral administration and the time of maximum plasma concentration (t_{max}) occurs within 1 to 2 hours in humans (178). AT tablets are 95 to 98% bioavailable and have a half-life ($t_{1/2}$) from 13 to 30 hours in humans (179).

Studies by Black et al. (180) in rodents found that AT, like other HMG-CoA reductase inhibitors, is relatively well absorbed with minimal systemic exposure. Lipid soluble statins, such as simvastatin and AT, enter enterohepatic cells easily (181). AT undergoes a large first-pass effect that produces pharmacologically active metabolites, which are recirculated via the bile to the liver, the target organ, and efficacy is enhanced, while the duration of action is prolonged. Thus, many HMG-CoA reductase inhibitors such as AT can provide a long and effective duration of action because biliary recycling occurs and the active metabolites are also as potent as AT itself (180). The t_{max} and $t_{1/2}$ in female rodents ranged from 1 to 5 hours and 2.5 to 5 hours respectively, using the 20, 100 and 225 mg/kg doses (174;176).

1.9 Rationale

In most organs, tissue damage results in a regenerative response, but in the pancreas, this regenerative capacity seems to be limited. Neovascularization is thought to be important in tissue regeneration after injury (182;183), and β-cell injury appears to be a potent stimulus for the recruitment of EPCs (96). Therefore, the interaction of the pancreas and vasculature could aid in the process of β-cell regeneration through EPCs, which can readily home to sites of injury and contribute to new blood vessel formation by delivering the proper signals for further tissue regeneration (96).

Taken altogether, it seems that bone marrow harbours EPCs, which could be important mediators in response to β-cell injury. In an attempt to manipulate EPC abundance in late fetal and neonatal life, AT was used because statins are known to mobilize EPCs in other tissues (138). We used a STZ model to experimentally induce β-cell injury and AT administration in order to effect the mobilization of EPCs which could potentially aid in the regeneration process through neovascularization (Figure 1.4).

1.10 Objectives

The first objective of this study was to examine the effect of AT on β-cell growth in a normal, uninjured pancreas. Our second objective was to examine the effects of AT after an STZ-induced injury to the β-cells in the neonatal rodent. Our third objective was to determine the effect of AT on neovascularization of the regenerating pancreas.

1.11 Hypothesis

We hypothesize that the rate and capacity of the β-cell growth and regenerative processes will be enhanced with an increasing AT dose, and that EPCs could be involved in neovascularization which will influence β-cell replacement.

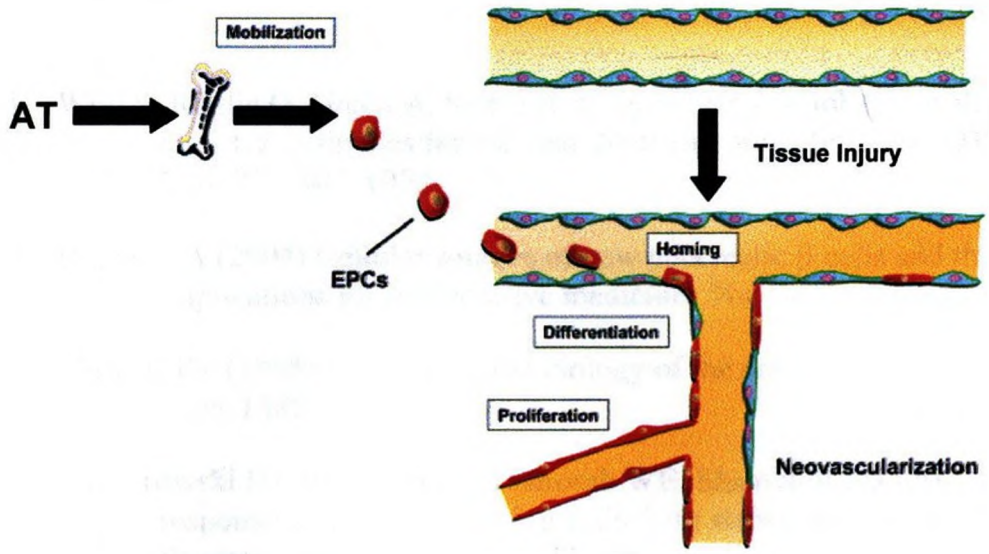

Figure 1.5. AT augments the EPC population and causes mobilization into peripheral blood where EPCs receive signals to home to sites of injury and contribute to neovascularization after STZ-induced injury in the neonatal rodent (Adapted from 184).

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CHAPTER 2

INTEGRATED ARTICLE

2.1 Introduction

The prevalence of diabetes is increasing at epidemic proportions and it is estimated that at least two hundred million individuals currently suffer from the disease (1;2). Due to deficient β-cell function, individuals with diabetes are unable to produce or secrete insulin in response to increasing blood glucose. Although insulin regimes can improve blood glucose levels, exogenous insulin administration cannot ensure continuous blood glucose control and prevention of diabetic complications (3-5). Due to the fact that islet transplantation has not been shown to be optimally effective, a considerable effort is being made to identify alternative sources of glucose-responsive, insulin-secreting cells for β-cell replacement, whether it be generating β-cells *in vitro* for transplantation or understanding how to expand or regenerate the β-cell population *in vivo* (6-8).

It is known that the β-cell mass is dynamic, and holds potential for targeted islet β-cell regeneration (9). Previously, we have used STZ to induce damage to β-cells of neonatal rodents and have studied the resulting β-cell regeneration process (10). A 70 mg/kg dose of STZ administered to neonatal rats at PD 4, resulted in destruction of approximately 60% of the β-cells in the pancreatic islets within 72 h and caused temporary hyperglycaemia that was returned to normal physiological range within 40 d post-STZ treatment (10). The changes seen in the damaged pancreas were an exaggeration of that which occurs during normal islet remodelling seen in rodents at this time. The β-cell destruction by STZ was rapidly reversed with β-cell mass having returned to 70% of control values by PD 40. The growth was due to both a re-population from within islets

and from neogenesis at the pancreatic ducts (10). Unfortunately, the mechanisms by which β-cells can regenerate are still poorly understood.

The vasculature may have a role in this regeneration process as the pancreas and the vasculature show a strong interaction starting early in development and maintain this interaction throughout islet formation. Lammert et al. (11) established that the blood vessel endothelium induces endocrine pancreatic differentiation, while the blood vessels provide metabolic sustenance for endocrine cell development and growth. The interaction of blood vessels with endocrine cells continues when mature hormone-secreting islets are formed. The islets are well vascularized throughout life which is critical for metabolic sensing and homeostatic regulation mediated by these cells (11). Circulating EPCs were identified and implicated in postnatal angiogenesis (12-14). They are detectable in the blood and bone marrow throughout life, although a decreasing number of circulating EPCs are seen with aging (15;16). Tissue injury and ischemia are potent stimuli for neovascularization (17).

Recently several studies demonstrated a novel function for statins, showing that they contribute to postnatal neovascularization by augmenting the mobilization of bone marrow-derived EPCs (18-20). Vasa et al. (18) demonstrated that the effects of statins on EPCs include increasing the number of circulating cells, reducing senescence, and enhancing proliferation and differentiation. Statin therapy in patients with CAD improved EPC differentiation into cardiomyogenic cells (21). Thus, the therapeutic use of autologous EPCs may stimulate cardiomyocyte regeneration, and thereby could potentially contribute to organ-specific regeneration generally (21).

In order to investigate the role of statins in pancreatic regeneration, we used a model of STZ-induced β-cell injury, and attempted to mobilize EPCs using AT, in order

to aid in β-cell recovery. The recruitment of EPCs, which readily home to sites of injury in other tissues, and potentially β-cell injury, would contribute to new blood vessel formation and deliver appropriate signals for β-cell regeneration. We hypothesize that the rate and capacity of the β-cell growth and regenerative processes will be enhanced with an increasing AT dose, and that EPCs could be involved in neovascularization which will influence β-cell replacement.

2.2 Materials and Methods

2.2.1 Animals

2.2.1.1 Breeding

Wistar rats were purchased from Charles River Laboratories (Montreal, PQ, CA). The animals were housed in the animal facilities at the Lawson Health Research Institute (London, ON, CA) in a temperature and humidity controlled room with a 12 h dark and 12 h light cycle. The rats had access to water and standard rat chow *ad libitum* and were able to acclimatize for a minimum of 72 h before any procedure was performed. Nulliparous female rats weighing 250-300 g were mated on the night of proestrus. The presence of sperm in the vaginal smear or a vaginal plug was confirmed the next morning, and the rat was considered pregnant, gestation day 0 (GD 0).

2.2.1.2 Gavage and STZ administration

Beginning at GD 15, the pregnant rats were given a daily oral gavage with a dose of either 20 or 40 mg/kg body weight dose of AT (Pfizer Canada Inc., Kirkland, PQ, CA) suspended in 0.5% carboxymethylcellulose (CMC) for 3 wk until PD 14. Control rats received a dose of 0.5% CMC alone. Litters were reduced to ten pups at birth, five males and five females. On PD 4, six pups in each litter were given a single intraperitoneal (ip) bolus injection of 70 mg/kg STZ freshly prepared in citrate buffer (pH 4.5). STZ at this dose has been shown to destroy approximately 60% of the β-cells (10). The remaining 4 animals received a control injection with citrate buffer alone. Our experimental groups consisted of control animals, treated with 0, 20 or 40 mg/kg AT and STZ animals treated with 0, 20 or 40 mg/kg AT and the time points examined were PD 6, PD 14 and PD 44.

2.2.1.3 Body, pancreas and blood glucose measurements

Body and pancreas weights were determined for offspring at PD 6 (2 d post-STZ), PD 14 (10 d post-STZ), and PD 44 (40 d post-STZ) of age. At PD 6, the pups were subjected to a non-fasting blood glucose test in order to determine the effect of STZ on the β-cells, and at PD 14, the animals were fasted for 4 h to determine fasted blood glucose levels to identify the amount of glucose at baseline. In both cases, the tail vein was lanced (up to 2 µl blood) to measure blood glucose using the Ascencia™ *AUTODISC*™ test strips (Bayer Corp., Elkhort, IN, USA) that measure glucose values from 0.5 to 33 mM (10 to 600 mg/dL) in whole blood. The animals were then killed by decapitation (PD 6) or carbon dioxide (CO_2) asphyxiation (PD 14) and the pancreata were collected, weighed, and fixed for histology.

2.2.1.4 GTT

At PD 44, animals were fasted for 5 h and subjected to an intraperitoneal glucose tolerance test (ipGTT) to examine β-cell function. A bolus of glucose (2 g/kg body weight) was given and blood was collected from the tail vein at 0, 15, 30, 60 and 90 min for glucose measurements. Glucose levels were measured at each time point as stated above and following 90 min, the animals were sacrificed by CO_2 asphyxiation. The pancreata were collected and fixed for histology and insulin extraction.

In order to determine the differences between each treatment an analysis of the area under the curve was performed using fasting blood glucose level as the baseline at 0 min. The percent change of blood glucose was also determined:

Percent change = [blood glucose 0 min - 30 min]/ blood glucose 0 min * 100

All animal procedures were performed with the approval of the Animal Care Committee of the University of Western Ontario in accordance within the guidelines provided by the Canadian Council for Animal Care (Appendix 1).

2.2.2 HPLC/MS

2.2.2.1 Sample preparation

Blood was extracted from female rats that were treated with no AT or 20 mg/kg AT at 0, 30, 60 and 90 min after gavage by lancing the tail vein and collecting 500 µl of blood. The blood was extracted from newborn pups (PD 1) and PD 6 that were treated with no AT or 20 mg/kg AT by decapitation, and 2 to 3 animals were pooled in order to obtain 500 µl of blood. The blood was collected in BD Microtainer® tubes with a Dipotassium ethylenediaminetetraacetic acid (EDTA) additive (BD Diagnostics, Franklin Lakes, NJ, USA) in order to extract the plasma. The tubes were spun at 3500 revolutions per minute (RPM) for 10 min and the plasma was removed and frozen.

The HPLC/MS analysis was done by Scott Bailey of Dr. David Freeman's laboratory. In order to prepare the sample for analysis, the Octadecyl (C18) Speedisk Column (10 µm, solid phase extraction (SPE)) (VWR International, Mississauga, ON, CA) were placed on an extraction box so waste could be collected for disposal. The tubes were conditioned by adding 1 ml methanol (MeOH) and 2 ml of deionized water (dH_2O) in order for the sorbent packing to interact properly with the compound of interest. Then 200 µl of water (H_2O), 100 µl of AT (sample), and 100 µl of rosuvastatin (RS) (1µg/ml), the internal standard, were added to the column which was then filled to the 1 ml mark with dH_2O. Positive pressure was applied to gently ease the sample through the extraction tube. The AT and RS bound to the sorbent packing, along with some impurities, and the liquid waste was pushed though. Impurities were removed with a

wash solvent (2 ml H_2O and then 1 ml 10% MeOH: H_2O with acetic acid (100 µl/200 ml)). Finally the AT and RS were eluted with 500 µl of MeOH into a glass vial which was dried down on an evaporator. The samples were then reconstituted in 100 µl 50:50 MeOH: H_2O in preparation for analysis.

2.2.2.2 HPLC/MS profile

HPLC/MS was used in order to determine the amount of parent compound AT that was present in female rats and pups at birth. The samples were separated by HPLC and analyzed by MS using a Thermo/Finnigan Surveyor HPLC with MS PUMP and Autosampler connected to Thermo/Finnigan Triple Stage Quadrupole (TSQ)-7000 Tandem MS in Electrospray Ionization (ESI) and Xcalibur Version 2.0 computer software (Thermo Electron Corp., Mississauga, ON, CA). The autoinjector extracted 10 µl of sample and inserted it into the flow leading to the analytical Luna C18 (2) column (3 µm, 100 Å, 50 x 3.0 mm) (Phenomenex, Inc., Torrance, CA, USA) at 40°C. In the column, the mobile phase consisted of A (54% acetonitrile (A.C.N.) with 5 mM ammonia formate buffer) and B (75% A.C.N. with 5 mM ammonia formate buffer). Components were eluted for 6 min beginning with 100% isocratic A, increasing to 100% B at 2.01 min and then decreasing to original concentration at 3.01 min, at a constant flow rate of 0.3 ml/min which pushed the sample through the column. The solid band of sample was then released from the column and introduced into MS using ESI, which eliminated the solvent from mobile phase and ionized the compounds for analysis.

Particles of interest were pulled into the vacuum tube at 300°C and then run through the quadrupoles (Q) where they were selectively identified as their mass + 1 due to ionization of the samples in the previous step. In the MS, the quadrupoles (Q1, Q2 & Q3) were used in order to specifically identify the compound of interest. The mass to

charge ratio (M/Z) for AT in positive mode for Q1 was 559 atomic mass units (amu), and then in Q2, the collision cell, AT went through the electric field at 25 electron Volts (eV) and was broken down into daughter compounds which were analyzed at a M/Z of 440 amu in Q3. It was important to note that no compounds would be broken down exactly the same and this was what made Q3 highly sensitive for identifying the correct compound. For our internal standard, RS, in positive mode, Q1 was 482.1 amu, and in Q2 it was fragmented at 30 eV to 258 amu, identified in Q3. The detector sent the information to the computer for processing. AT was identified based on its retention time of 2.6 min compared with the internal standard, RS (1000 ng/ml) at 1.5 min. The chromatogram (Appendix 2) identified the relative abundance of AT and RS at their respective retention times.

2.2.3 Immunohistochemistry

Pancreatic tissue was fixed in 10% neutral buffered formalin (VWR, West Chester, PA, US) for 24-36 h and embedded in paraffin (University Hospital Pathology Lab, London, ON, CA). Ten consecutive tissue sections of 5 μm were cut with a Leica Rotary Microtome (Leica Microsystems, Richmond Hill, ON, CA), mounted on SuperFrost® Plus glass slides (Fisher Scientific, Ottawa, ON, CA), and incubated at 50°C for 24 h.

2.2.3.1 Glucagon/Insulin

Dual staining was performed on pancreas tissue sections to localize the presence of α-cells with mouse monoclonal anti-glucagon raised in mouse (Sigma-Aldrich, St. Louis, MO, USA) and β-cells by using a rabbit polyclonal anti-insulin raised against insulin of human origin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by a modified avidin-biotin-peroxidase method (Hsu et al., 1981). All slides were deparaffinized and

rehydrated through a series of xylene (EMD Chemicals, Gibbstown, NJ, USA) and ethanols (EtOH) (100%, 90%, 70%) (Fisher Scientific, Ottawa, ON, CA). Slides were then incubated in 3% (vol/vol) hydrogen peroxidase (H_2O_2) (Fisher Scientific, Fair Lawn, NJ, USA) for 10 min to block endogenous peroxidase activity. The slides were washed two times in 1X Dulbecco's Phosphate Buffered Saline (PBS) (pH 7.2-7.6) (Sigma-Aldrich, St. Louis, MO, USA) after each step excluding the blocking step. Slides were put into a humidified chamber and unless otherwise stated, all incubations and washes were performed at room temperature (RT). A block containing 5% (vol/vol) normal horse serum (Cedarlane Laboratories Ltd., Hornby, ON, CA), 2% (wt/vol) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA), and PBS, was used to reduce non-specific, background staining and applied for 30 min. All antisera were diluted in DakoCytomation Antibody Diluent (AD) (DakoCytomation, Mississauga, ON, CA). The primary antibody, anti-glucagon (1:2000 dilution) was incubated for 1 h and then biotinylated horse anti-mouse (Vector Laboratories Inc., Burlingame, CA, USA) was used as the secondary antibody (1:30 dilution) and incubated for 30 min. Slides were incubated with ExtrAvidin peroxidase (Sigma-Aldrich, St. Louis, MO, USA), diluted with AD, for 30 min and then developed in fresh diaminobenzedine (DAB) (BioGenex, San Ramon, CA, USA) for 1 to 5 min to visualize glucagon positive areas in brown.

A block, to eliminate non-specific binding, was prepared with goat serum (Cedarlane Laboratories Ltd., Hornby, ON, CA) and incubated as explained above. This was followed by incubation with the primary antibody, anti-insulin (1:200 dilution) overnight at 4°C. Biotinylated goat anti-rabbit (Vector Laboratories Inc., Burlingame, CA, USA) was used as a secondary antibody (1:30 dilution) and incubated for 30 min. The Vectastatin® ABC-AP Kit and then Vector® Red Alkaline Phosphatase Substrate Kit

I were incubated for 30 min (Vector Laboratories Inc., Burlingame, CA, USA), with the addition of levamisole (Vector Laboratories Inc., Burlingame, CA, USA) which blocked endogenous alkaline phosphatases. Insulin-positive cells were visualized in red after incubation for up to 30 min in the dark. Tissue sections were counter-stained with Carazzi's hematoxylin, prepared with hematoxylin (BDH Inc., Toronto, ON, CA), glycerol (Fisher Scientific, Fair Lawn, NJ, USA), aluminum potassium sulphate (Fisher Scientific, Fair Lawn, NJ, USA), potassium iodate (Fisher Scientific, Fair Lawn, NJ, USA), and dH_20. The slides were rehydrated in a series of EtOH (50%, 70%, 90%, 100%) and xylene, and then mounted with Permount® (Fisher Scientific, Fair Lawn, NJ, USA) and a glass coverslip.

To establish specificity of the antibodies, controls were prepared by way of substituting the primary antibody with non-immune serum, secondary antiserum omission, and the absence of staining after pre-incubation of the primary antisera with excess antigen. For the final control, the last positive dilution for each antiserum was determined and 5 times the molar concentration of the respective peptide was added. After an overnight incubation at 4°C, the antibody-peptide complex was centrifuged at 14 000 RPM for 30 min, and the supernatant was collected. An absence of signal using the supernatant as a primary antibody indicated antibody specificity.

2.2.3.2 Flk1/BS-1 lectin

Dual staining was performed on pancreas tissue sections to localize the presence of islet ECs using mouse monoclonal anti-Flk1 raised in mouse (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and biotinylated BS-1 lectin (Vector Laboratories Inc., Burlingame, CA, USA) as previously described (22). All slides were deparaffinized and rehydrated through a series of xylene and ethanols. The slides were then subject to

heat induced epitope retrieval (HIER) with 0.01 M citrate buffer (pH 6.0), which was prepared with citric acid monohydrate (Sigma-Aldrich, St. Louis, MO, USA), dH_20 and sodium hydroxide (NaOH). The HIER was used to unmask the epitope which became altered due to protein cross-linking during formalin fixation. The slides were placed in citrate buffer in the Decloaking chamber (Biocare Medical, Walnut Creek, CA, USA). It was heated to 95-100°C and the slides incubated at this temperature for 20 min. The slides were then removed and placed at RT to cool for 20 min. 10X Tris Buffered Saline-Tween 20® (TTBS) (pH 7.6) was prepared using a 0.5 M Tris Base (VWR Int., Mississsauga, ON, CA), 9% (wt/vol) sodium chloride (NaCl), 0.5% (vol/vol) Tween 20® (Sigma-Aldrich, St. Louis, MO, USA), and concentrated hydrochloric acid (HCl) (EMD Chemicals, Inc., Gibbstown, NJ, USA). 1X TTBS was used as a wash after each step excluding the block. A block (horse) for non-specific staining was prepared as mentioned above and applied for 30 min. Again, all antisera were diluted in AD. The primary antibody, anti-flk-1 (1:50 dilution) was incubated for 1 h and then incubated in 3% (vol/vol) H_2O_2 and then biotinylated horse anti-mouse was used as secondary antibody (1:30 dilution) and incubated for 30 min. The Vectastatin® Elite® ABC Kit and then Vector® SG Substrate Kit for Peroxidase were incubated for 30 min and then 5-10 min respectively (Vector Laboratories Inc., Burlingame, CA, USA), to visualize the Flk1-positive areas in grey.

Biotinylated BS-1 lectin (1:30 dilution) was incubated overnight at 4°C. The Vectastatin® ABC-AP Kit and then Vector® Red Alkaline Phosphatase Substrate Kit I were incubated for 30 min to visualize the BS-1 lectin-positive areas in red. Tissue sections were counter-stained with methyl green solution (Sigma-Aldrich, Milwaukee, WI, USA), which was prepared with acetic acid glacial (BDH Inc., Toronto, ON, CA) and

dH_20. Slides were then rehydrated in EtOH (90%, 100%) and xylene, and then mounted with Permount® and a glass coverslip.

To establish specificity of the antibodies, controls were prepared by way of substituting the primary antibody with non-immune serum, or secondary antiserum omission.

2.2.4 Morphometric analysis

Morphometric analysis was performed on pancreata from five to six animals from each age and treatment group. One section per animal was analyzed after doing a validation study to establish that this was representative (Figure 2.1A-C).

Analysis of the pancreas sections was performed using a Carl Zeiss Axioskop transmitted light microscope (Carl Zeiss, Inc., New York, NY, USA) with QImaging MicroPublisher 3.3 Real Time Viewing (RTV) camera (QImaging, Burnaby, BC, CA). Digital pictures were taken from slides of the entire tissue with the 2.5X objective and of each individual islet or α-cell cluster with the 40X, 20X or 10X objective depending on the size. Image analysis was performed with Northern Eclipse Version 7.0 morphometric analysis software (Empix Imaging, Inc., Mississauga, ON, CA). Pictures of the micrometer slide were captured with the Zeiss microscope using each of the objective lenses (2.5X, 10X, 20X and 40X) and matched with an electronic calibration bar in order to determine the correct calibration of the microscope. The pixel measurement was calibrated to micrometres (Appendix 3).

An islet was defined as containing at least two cells, including one insulin-positive cell, having a total area of 200 μm^2 or greater. Islets were arbitrarily separated by area into small (200-5000 μm^2), medium (5000-10 000 μm^2) and large (>10 000 μm^2). An α-cell cluster was defined as containing at least two glucagon-positive cells, having a total

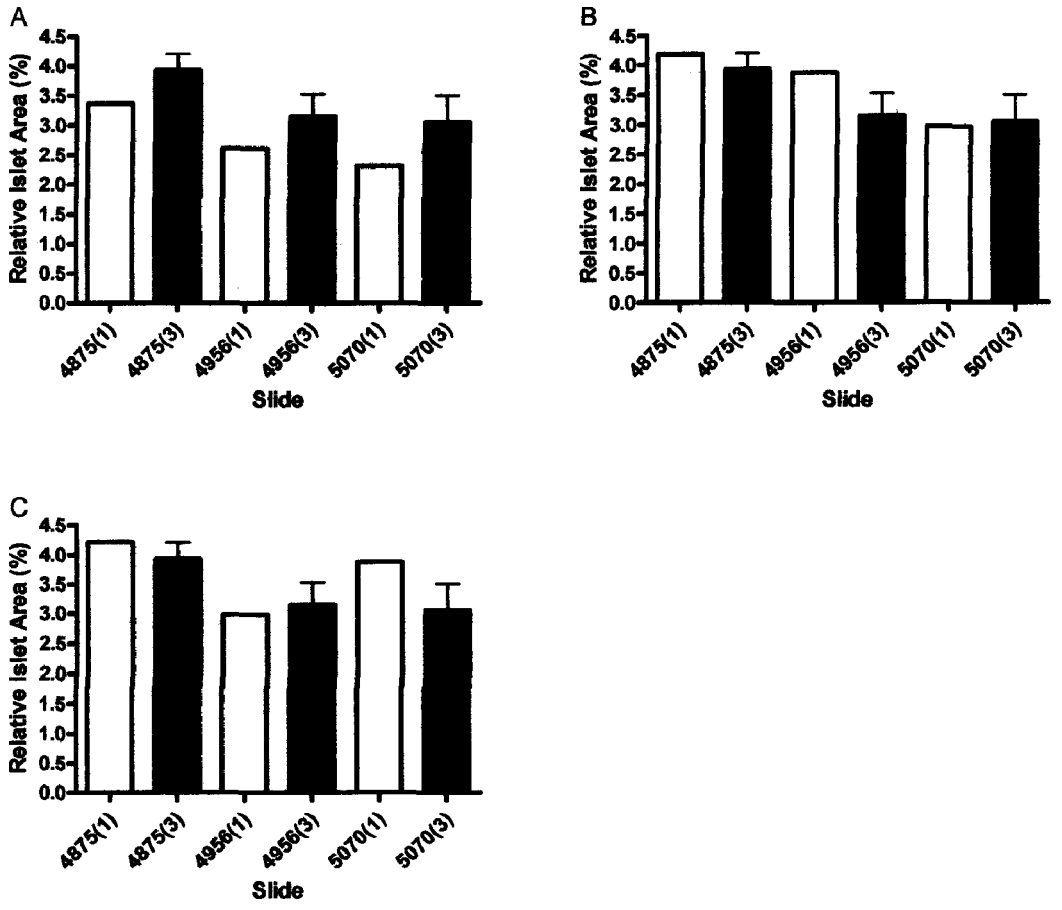


Figure 2.1. In order to verify that analysis of a single pancreatic section per animal was representative of the pancreas, an average of 3 sections were examined and the relative islet area (per whole tissue) was plotted in 3 control animals at PD 14. Each animal is indicated by a different tissue section number. The value in parentheses is the number of slides analyzed in each tissue. When 3 slides were analyzed, they were cut 50 μm apart. Each graph (A-C) represents the relative islet area (%) of 1 slide versus the average of 3 slides from the same animal. Figures represent mean ± SEM derived for each tissue that had 3 slides analyzed. We determined it was more beneficial to increase our n value versus an analysis of repeated sections per tissue.

area 200 μm^2 or greater, but less than 5 000 μm^2. For each pancreatic section, the following were determined: the total area of the pancreatic section, the area occupied by each islet, and the area occupied by the α-cells (glucagon-positive) and β-cells (insulin-positive) within each islet, the total number of islets, and the area of each α-cell cluster. The perimeter of the tissue or cell was traced and the area (μm) was calculated by the computer software based on the total number of pixels that an object occupied. The following calculations were determined:

β-cell mass = total β-cell area/ total pancreatic tissue area * pancreas weight

Islet density (total, small, medium, large) = number of islets (total, small, medium, large)/ total pancreatic tissue area

α-cell cluster relative area = total α-cell cluster area/ total pancreatic tissue area *100

As well, the area of individual β-cells and exocrine cells was determined. The area of individual β-cells was determined by two blinded observers who choose ten random islets from each pancreatic section and traced the individual β-cells. The average β-cell area was then calculated for ten islets. The individual exocrine cell area was determined in the same way.

To estimate the amount of Flk1 and BS-1 lectin-positive ECs, two blinded observers selected 15 random islets from each pancreatic section and counted all the Flk1-positive cells, BS-1 lectin-positive cells, and Flk1/ BS-1 lectin dual positive cells for each islet. The data of both blinded observers was then averaged for all calculations.

2.2.5 Insulin extraction and RIA

Pancreatic insulin was extracted in acid ethanol (165mM hydrochloric acid (HCl) in 75% EtOH) (23). A 100 mg head portion of the pancreas was homogenized in 1 ml acid ethanol (0.01 ml/mg) and the homogenate was left overnight at 4°C. The

homogenate was then centrifuged at 2000g for 5 min at 4°C and the supernatant was removed and stored at -20°C until analysis of insulin content.

Insulin content in the pancreas and serum was measured using a Sensitive Rat Insulin RIA Kit (Linco Research, Inc., St. Charles, MO, USA). The pellets were counted 1 min/tube using the Cobra II Auto-Gamma Counter (Packard). Standards and quality controls were analyzed. The dose of the standards were known and therefore a logit-log curve could be created for the mathematical treatment of the data (Appendix 4). The insulin content was normalized to mg of pancreatic tissue. The assay sensitivity was 3 pM and the inter- and intra-assay coefficients of variation were 4% and 9% respectively.

2.2.6 Statistical analysis

Data was represented as mean ± SEM and was compared using a two-way ANOVA to assess the statistical difference between treatments, followed by a Duncan's multiple range test when interaction was present. If there was no combined effect of the treatments, each treatment group (Control or STZ; with or without AT) was compared separately using a using a one-way ANOVA with a Newman-Keuls multiple comparison test. Differences were considered statistically significant at $p<0.05$. Statistical analysis was performed using GraphPad Prism Version 4 (GraphPad Inc., San Diego, CA, USA). Males and females had similar trends so data was combined. Four to ten animals from each age and treatment group were examined for each experimental procedure.

2.3 Results

2.3.1 Islet morphology after STZ treatment

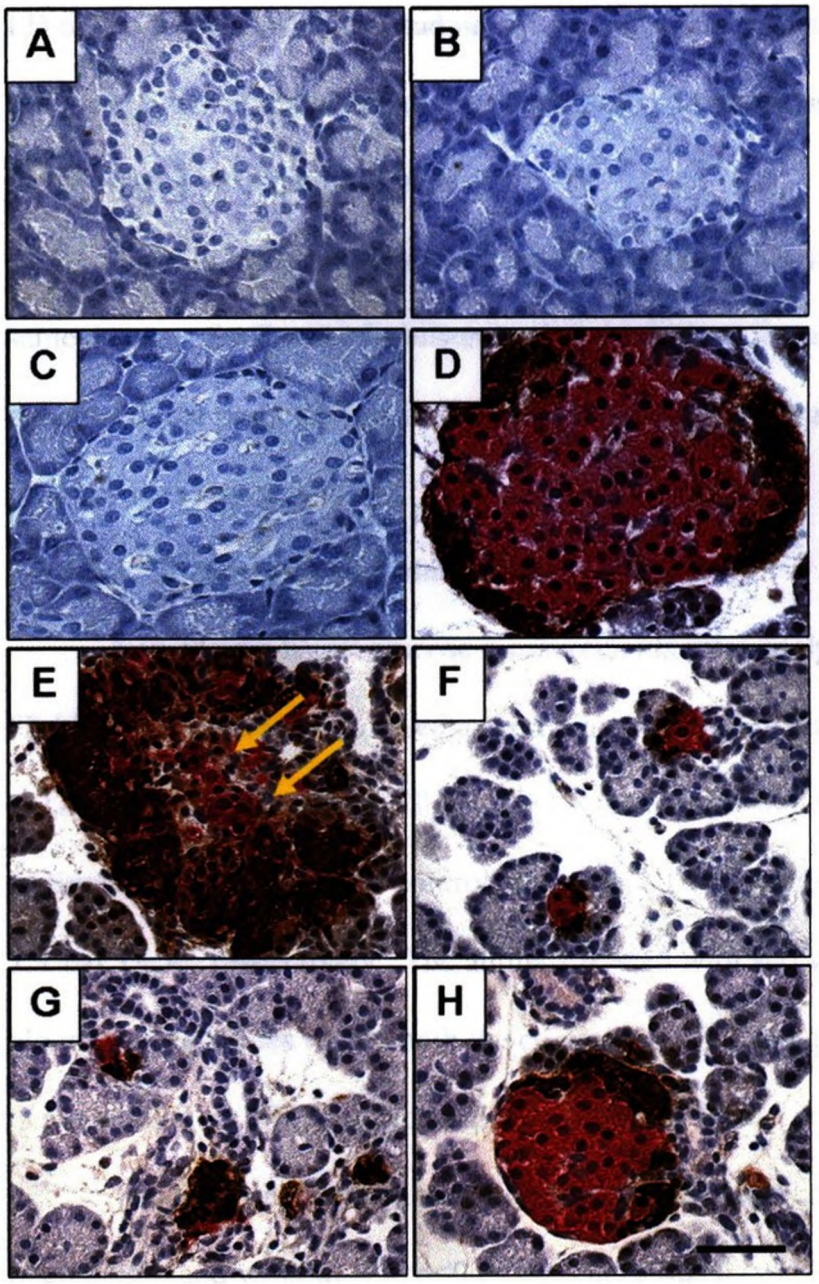
Histological analysis illustrated that β-cell mass was reduced after STZ-induced damage at PD 6 (2 d post-STZ) (Figure 2.2E) compared to control animals at the same age (Figure 2.2D). At PD 14 (10 day post-STZ), we found small insulin positive cell clusters (Figure 2.2F) often in association with pancreatic ducts (Figure 2.2G). By PD 44, no histological evidence of β-cell damage was seen within the islets (Figure 2.2H). Negative controls were prepared in order to determine antibody specificity (Figure 2.2A-C).

β-cell mass was significantly decreased in the STZ-treated animals ($p<0.001$) at PD 6, PD 14 and PD 44 compared to the controls at the same age (Figure 2.2I). β-cell mass was significantly reduced from 2.07 ± 0.19 mg in control animals to 0.53 ± 0.10 mg in STZ-treated animals ($p<0.001$) at PD 6 (2 d post-STZ treatment). This 75% β-cell loss at PD 6 of age was similar to that previously reported (10). β-cell mass in the control animals increased two-fold from PD 6 to PD 44, while in the STZ-treated animals, β-cell mass increased four-fold from PD 6 to PD 44. Although, the STZ-treated animals showed a larger incremental increase in β-cell mass, the β-cell mass of STZ-treated animals was still only half of the controls at PD 44 (Figure 2.2I).

2.3.2 Administration of AT during gestation

In order to determine if the AT administered to the mother during gestation crossed the placenta and was present in the blood stream of the pups, we measured the concentration of parent AT at PD 1 (birth) and PD 6. The offspring of mother's who did not receive AT had no AT present in their plasma, while the offspring of mother's who received AT had 11.1 ± 3.1 ng/ml AT present in their plasma at PD 1 ($n=10$). The pups at

Figure 2.2. Micrographs depicting immunohistochemical localization of insulin (red) and glucagon (brown) in rat pancreata (A-H). Negative controls with no primary antibodies (A), no secondary antibodies (B) and pre-absorption of primary antibody with excess insulin and glucagon peptide (C). Islets of control (D) and STZ-treated (E) animals are shown at postnatal PD 6. *Yellow arrows* indicate β-cell loss after STZ (E). Ten days after STZ treatment (PD 14), small insulin positive cell clusters were seen (F) and frequently in association with pancreatic ducts (G). By PD 44, no evidence of remaining β-cell injury was apparent within the islets (H). Scale bar=50 μm. β-cell mass at PD 6, PD 14 and PD 44 (I) in control (white bars) and STZ-treated (black bars) animals. The reduction in β-cell area was approximately 75% at PD 6 (2 d post-STZ treatment). Figure represents mean ± SEM derived from 5-6 animals from different litters. ***$p<0.001$ vs. control at the same age by a one-way ANOVA with a Newman-Keuls Multiple Comparison Test.


A
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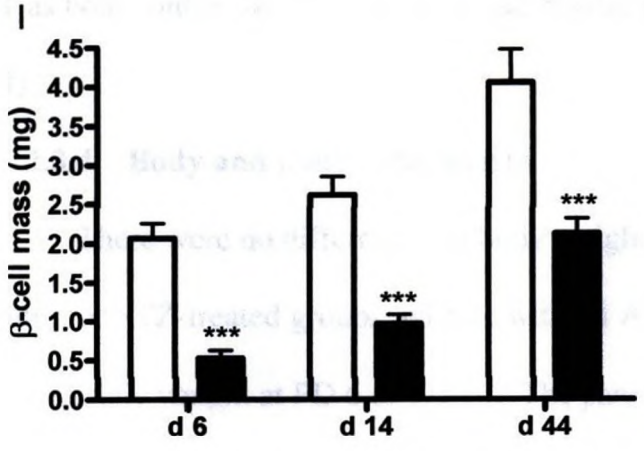

I
β-cell mass (mg)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
d 6
d 14
d 44

PD 6 who had received AT, had no AT present in their plasma due to the fact that only AT metabolites could be measured at this time and we did not have access to these metabolites for comparative analysis.

Also, in order to understand the pharmacokinetics of AT in the adult female, the same dosage of AT was administered to non-pregnant females who were age and weight-matched to the pregnant rats in our study. The peak concentration of AT was seen 60 min post-gavage (Figure 2.3)

2.3.3 β-cell mass and blood glucose at PD 6

Non-fasting blood glucose measurements were analyzed on PD 6 (2 d post-STZ) (Table 2.1). The results showed a significant increase in both the STZ groups with AT (12.4± 1.3 mM) or without AT (11.7 ± 1.3 mM) compared to controls ($p<0.001$). Non-fasting blood glucose in the control rats, with AT and without AT were 7.4 ± 0.5 mM and 7.9 ± 0.3 mM, respectively. The animals treated with and without AT showed similar blood glucose values within both the control and STZ groups, indicating that the AT treatment had no effect on blood glucose levels at PD 6.

The STZ treatment caused a decrease β-cell mass compared to controls in animals treated with and without AT ($p<0.001$). There was no evidence of a protective effect of AT as both control and STZ animals had similar β-cell mass with or without AT (Figure 2.4).

2.3.4 Body and pancreatic weight

There were no differences in body weight at PD 6, PD 14 or PD 44 in either the control or STZ-treated groups, with or without AT. Similarly, there were no differences in pancreatic weight at PD 6 or PD 14. The pancreatic weight at PD 44 in the control with 40 mg/kg AT (1580 ± 81 mg) group was significantly higher than the control

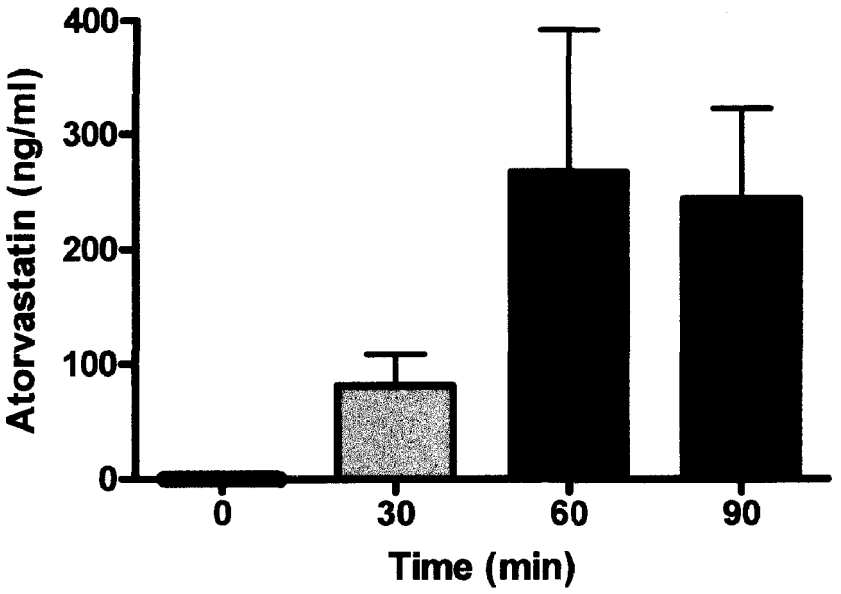


Figure 2.3. The concentration of AT parent compound detected in adult female rats by HPLC/MS. The rats received a gavage of 20 mg/kg dose of AT at 0 min and plasma was collected in order to determine the concentration of AT at 0 min, 30 min (light grey bar), 60 min (dark grey bar), and 90 min (black bar). Figures represent mean ± SEM derived from 3 adult females from different litters.

Table 2.1. Non-fasting blood glucose measurements at PD 6 (2 days post-STZ) in both control and STZ-treated animals, treated with either a dose of 0 or 20 mg/kg AT.

		AT Dose (mg/kg)	
		0	20
Non-fasting Blood Glucose (mM)	Control	7.9±0.3	7.4±0.5
	STZ	11.7±1.3	12.4±1.3

Data are mean ± SEM; n=5-6 litters. STZ treatment is significant ($p<0.001$) by a two-way ANOVA.

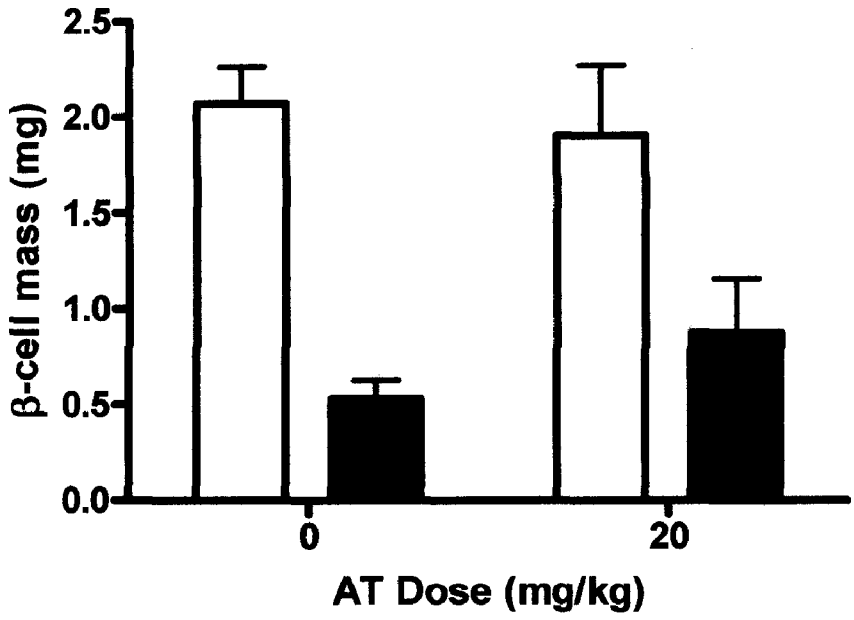


Figure 2.4. β-cell mass at PD 6 in both control (white bars) and STZ-treated (black bars) animals with a dose of 0 or 20 mg/kg AT. Figures represent mean ± SEM derived from 5-6 animals from different litters. STZ treatment was significant ($p<0.001$) by a two-way ANOVA.

Table 2.2. Body and pancreatic weight at PD 6, PD 14 and PD 44 in control and STZ-treated animals, treated with a dose of 0, 20 or 40 mg/kg AT.

			AT Dose (mg/kg)		
			0	20	40
Body Weight (g)	d 6	Control	15±0.5	13±0.8	–
		STZ	13±0.4	13±0.5	–
	d 14	Control	32±1	35±2	32±1
		STZ	31±1	33±1	32±2
	d 44	Control	199±16	206±13	201±11
		STZ	180±13	199±12	187±11
Pancreas Weight (mg)	d 6	Control	68±4	60±6	–
		STZ	66±4	69±5	–
	d 14	Control	218±20	158±21	166±12
		STZ	241±17	205±21	185±13
	d 44	Control	1106±60	1180±8	1580±81##
		STZ	1194±93	1274±81	1599±87*

Data are mean ± SEM; n=5-6 animals from different litters/group. ##$p<0.01$ vs. d 44 control + 0 mg/kg and control + 20 mg/kg, *$p<0.05$ vs. d 44 STZ + 0 mg/kg and STZ + 20 mg/kg by a one-way ANOVA with Newman-Keuls Multiple Comparison Test.

animals without AT (1106 ± 60 mg) and 20 mg/kg AT (1180 ± 87 mg), respectively ($p<0.01$) (Table 2.2).

In the STZ + 40 mg/kg AT group, the pancreatic weight (1599 ± 87 mg) was also significantly larger compared to the STZ-treated animals without AT (1194 ± 9 mg) and 20 mg/kg AT (1274 ± 81 mg) ($p<0.05$) (Table 2.2).

2.3.5 β-cell mass and blood glucose at PD 14 and PD 44

β-cell mass was calculated in order to determine the effect AT on the growth and regeneration of the β-cells. In the animals that received STZ + 40 mg/kg AT, there was a significant decrease in β-cell mass at PD 14, when compared to animals that did not receive AT ($p<0.05$) indicating a potential harmful effect of the combined drugs at this dose (Figure 2.5A).

Correspondingly, fasting blood glucose values at PD 14 for the animals treated with STZ, both with AT (8.1 ± 0.9 mM) and without AT (8.3 ± 0.7 mM), were significantly higher than in the controls with AT (6.7 ± 0.3 mM) and without AT (6.9 ± 0.2 mM) indicating moderate hyperglycaemia ($p<0.05$; n=5-6). There was no effect of AT on fasting blood glucose levels (data not shown).

By PD 44, the β-cell mass of control + 20 mg/kg AT (5.56 ± 0.80 mg) animals was significantly greater than in animals that did not receive AT (4.05 ± 0.42 mg) ($p<0.05$). Additionally, the β-cell mass of STZ + 20 mg/kg AT (3.23 ± 0.33 mg) and STZ + 40 mg/kg AT (2.89 ± 0.12 mg) were both significantly increased when compared to animals that did not receive AT (2.13 ± 0.19 mg) ($p<0.05$) at PD 44 (Figure 2.5B).

The β-cell mass of the STZ-treated animals was increased two-fold from PD 14 to PD 44 in the STZ + 0 mg/kg AT, three-fold in the STZ + 20 mg/kg AT, and five-fold in the STZ + 40 mg/kg AT. The β-cell mass of the control-treated animals was increased

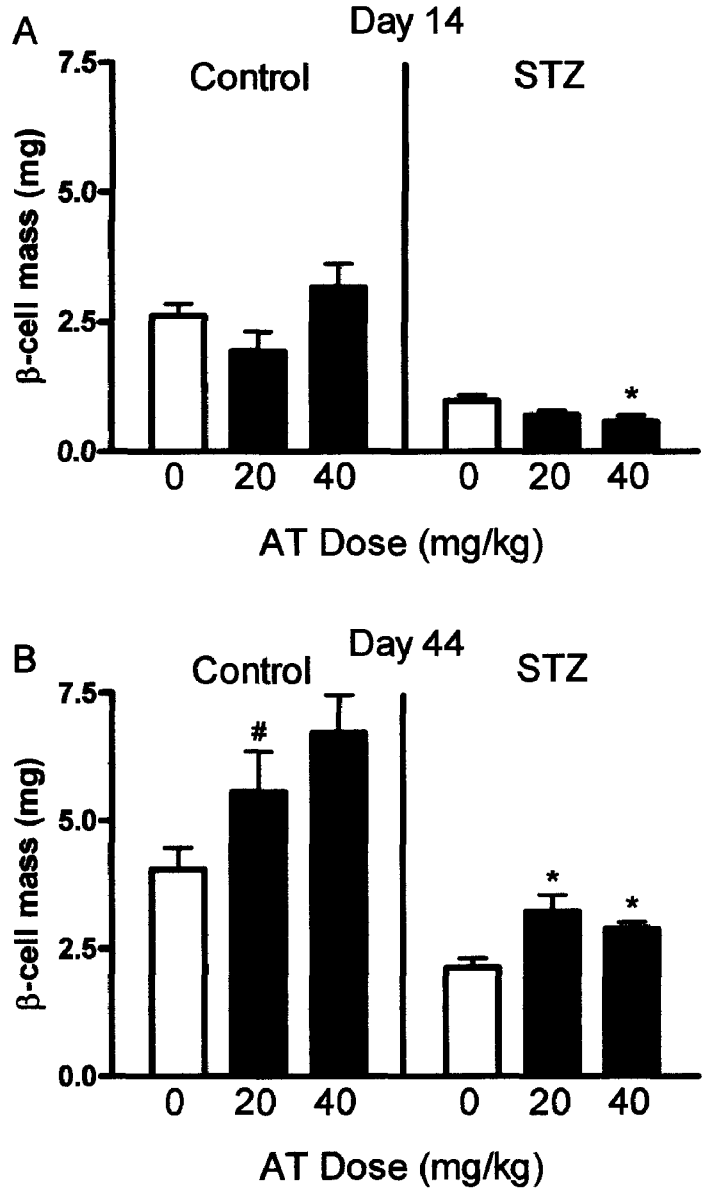


Figure 2.5. β-cell mass at PD 14 (A) and PD 44 (B) in both control and STZ-treated animals, with a dose of 0, 20 or 40 mg/kg AT. Control and STZ-treated animals were analyzed separately. The AT dose was represented as follows in both the control and STZ groups; 0 mg/kg (white bars), 20 mg/kg (black bars) and 40 mg/kg (grey bars). Figures represent mean ± SEM derived from 5-6 animals from different litters. *$p<0.05$ vs. STZ + 0 mg/kg AT; #$p<0.05$ vs. control + 0 mg/kg AT by a one-way ANOVA with a Newman-Keuls Multiple Comparison Test.

from PD 14 to PD 44 also, but to a lesser extent than the STZ-treated animals, except for the control + 20 mg/kg AT which increased three-fold. This increase was correlated with the dosage of AT at PD 44 (Figure 2.5B), but not at PD 14, where there was a decrease in β-cell mass in the STZ-treated animals (Figure 2.5A).

2.3.6 β-cell and exocrine cell size

In order to determine if the increase in β-cell mass was due to an increase in individual β-cell size, we examined β-cell area at PD 14 and PD 44. The individual β-cell size was increased at PD 14 in the control + 40 mg/kg AT (81 ± 2 μm^2) compared to no AT (73 ± 1 μm^2) ($p<0.05$) (Figure 2.6A). At PD 44, in the STZ group, the individual β-cell size in the 40 mg/kg dose was 82 ± 2 μm^2 and was significantly greater than the 0 mg/kg dose, 69±2 μm^2 ($p<0.001$) and 20 mg/kg dose, 72 ± 1 μm^2 ($p<0.01$) (Figure 2.6B). The β-cell hypertrophy that was seen in the STZ + 40 mg/kg AT dose could have contributed to the increased β-cell mass at PD 44.

There was no change in exocrine cell size at either PD 14 or PD 44 (Figure 2.7A and B).

2.3.7 Islet size distribution

Islet size distribution was expressed as numbers of total, small (200-5000 μm^2), medium (5000-10 000 μm^2) and large (>10 000 μm^2) islets per mm^2 of pancreas. At PD 14, there were no significant changes in total, small, medium or large islet distribution, although there were less large islets in the STZ group compared to the controls (Figure 2.8G). By PD 44, there was a significant increase in the total number of islets in the control + 20 mg/kg AT group, 1.42 ± 0.11 mm^2 ($p<0.01$) and the control + 40 mg/kg AT group, 1.26 ± 0.06 mm^2 ($p<0.05$) compared to no AT, 0.98 ± 0.04 mm^2 (Figure 2.8B). There was a similar increase in the STZ + 20 mg/kg AT group (1.67 ± 0.22 mm^2)

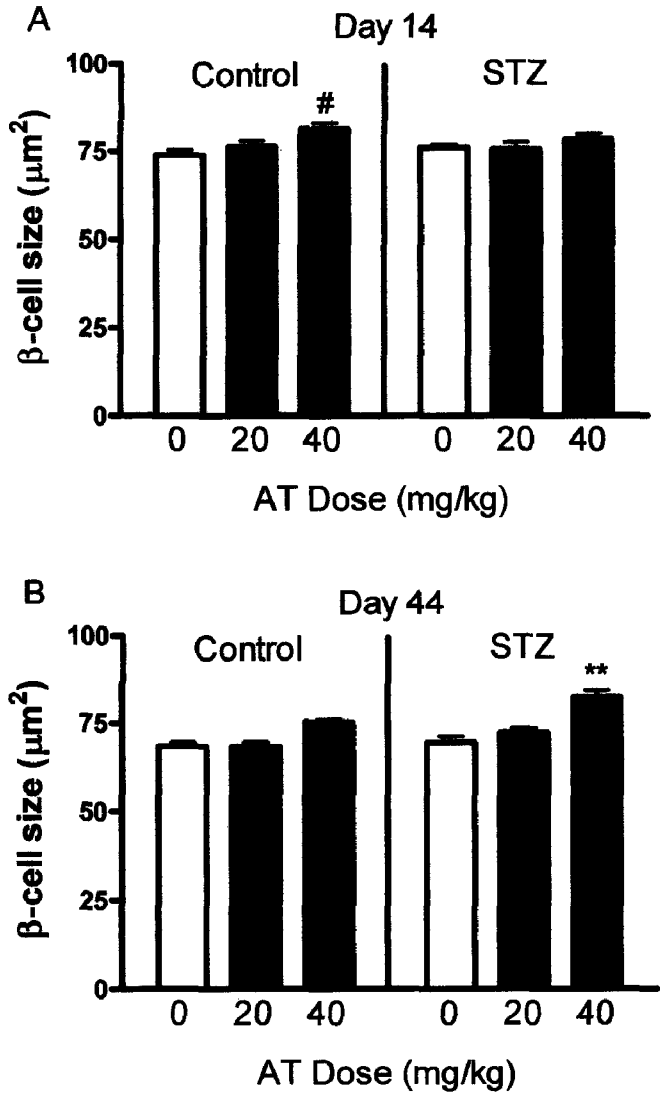


Figure 2.6. β-cell size at PD 14 (A) and PD 44 (B) in both control and STZ-treated animals, with a dose of 0, 20 or 40 mg/kg AT. Control and STZ-treated animals were analyzed separately. The treatment with AT was represented in both the control and STZ groups; 0 mg/kg (white bars), 20 mg/kg (black bars) and 40 mg/kg (grey bars). Figures represent mean ± SEM derived from 5-6 animals from different litters. #$p<0.05$ vs. control + 0 mg/kg AT; **$p<0.01$ or less vs. STZ + 0 mg/kg and 20 mg/kg AT, by a one-way ANOVA with a Newman-Keuls Multiple Comparison Test.

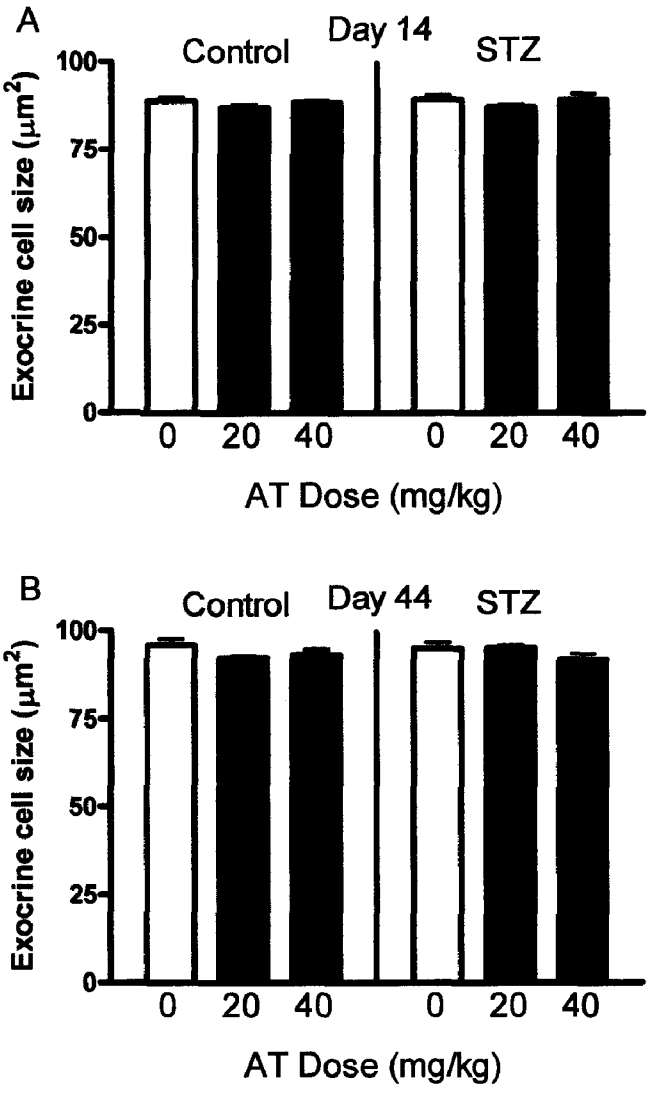


Figure 2.7. Exocrine cell size at PD 14 (A) and PD 44 (B) in both control and STZ-treated animals, and a dose of 0, 20 or 40 mg/kg AT. Control and STZ-treated animals were analyzed separately. The AT dose was represented as follows in both the control and STZ groups; 0 mg/kg (white bars), 20 mg/kg (black bars) and 40 mg/kg (grey bars). Figures represent mean ± SEM derived from 5-6 animals from different litters.

compared to animals that did not receive AT (1.09 ± 0.07 mm^2) ($p<0.05$), but not in the STZ + 40 mg/kg AT (Figure 2.8B). Corresponding to the changes in the total islets, there was an increase in the number of small islets per section in the control + 20 mg/kg (1.15±0.07 mm^2) ($p<0.001$) and 40 mg/kg (1.02 ± 0.05 mm^2) ($p<0.01$) AT groups compared to no AT (0.73 ± 0.04 mm^2) (Figure 2.8D). As well, there was a significant increase in the distribution of small islets STZ + 20 mg/kg AT group (1.45 ± 0.24 mm^2) ($p<0.05$) compared to animals who did not receive AT (0.96 ± 0.08 mm^2) (Figure 2.8D). By PD 44, there were less large islets in the STZ groups as compared to the controls (Figure 2.8H), but similar numbers of total, small, and medium islets.

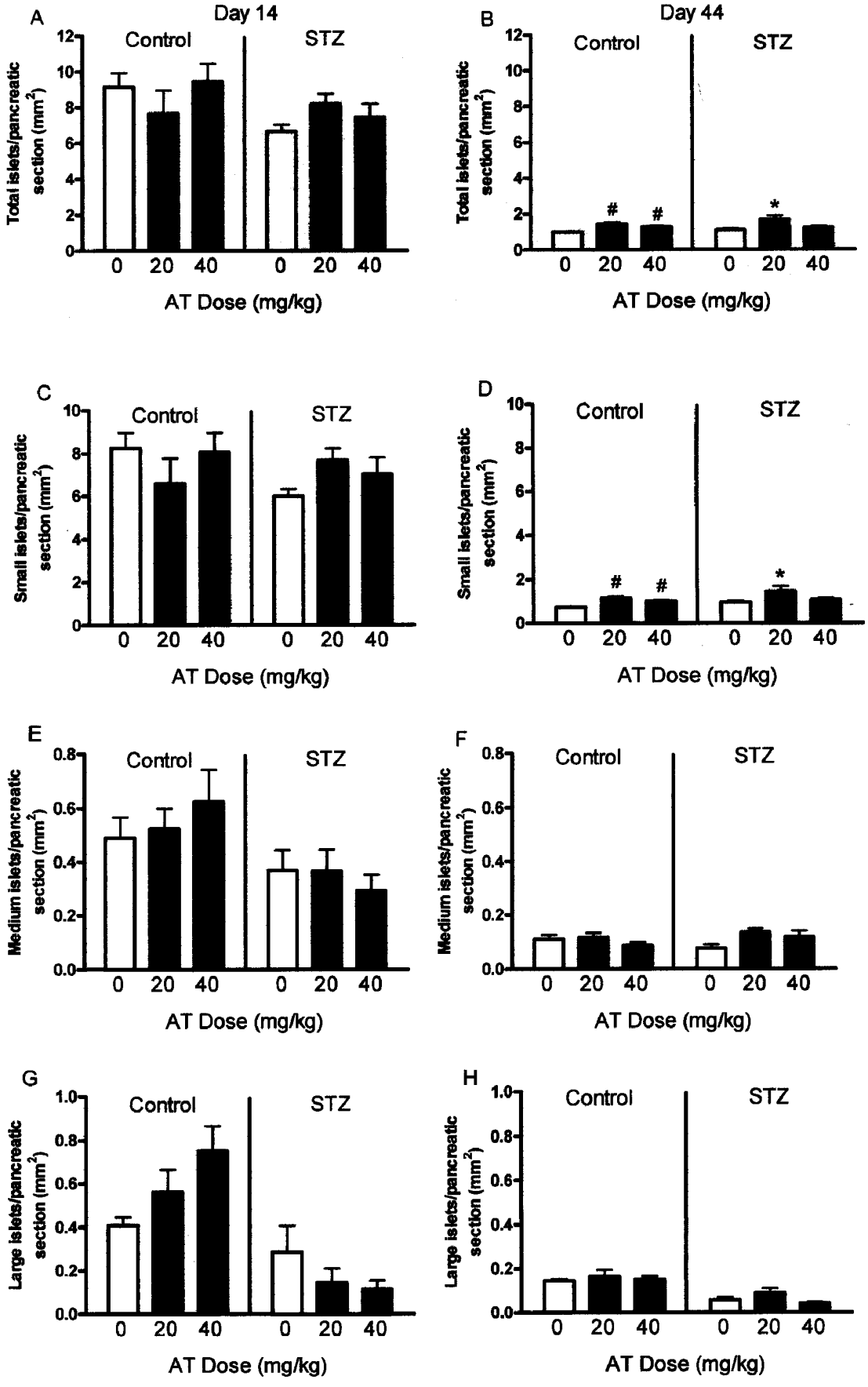
2.3.8 *α*-cell clusters

Morphometric analysis demonstrated that α-cell clusters were visible within the pancreas (Figure 2.9A) and budding off ducts (Figure 2.9B) and this was similar to our previous findings (10). At PD 14, there was a larger percentage of α-cell clusters per pancreatic section than at PD 44, where the occurrence of α-cell clusters was rare (Figure 2.9C and D). These clusters may contribute to the increased β-cell mass as primitive islets or release signals for proliferation of islets.

2.3.9 Glucose tolerance

Before administering the GTT at PD 44, fasting blood glucose values were measured in the animals at 0 min. The STZ + 40 mg/kg AT dose had a higher mean glucose than the control + 40 mg/kg AT dose ($p<0.05$), at 10 ± 0.9 mM and 6.9 ± 0.3 mM respectively (Table 2.3). The animals were then injected with a 2g/kg bolus of glucose. Blood glucose was measured and blood samples were collected at 0, 15, 30, 60 and 90 min to determine functionality of the islets. The blood glucose values at 30 min post-

Figure 2.8. Islet density distributed by size for total islets per pancreatic section at PD 14 (A) and PD 44 (B), small islets (200-5000 μm^2) per pancreatic section at (C) PD 14 and (D) PD 44, medium islets (5000-10 000 μm^2) per pancreatic section at (E) PD 14 and (F) PD 44, and large islets (>10 000 μm^2) per pancreatic section at (G) PD 14 and (H) PD 44 in both control and STZ-treated animals, treated with a dose of 0, 20 or 40 mg/kg AT. Control and STZ-treated animals were analyzed separately. The AT dose was represented as follows in both the control and STZ groups; 0 mg/kg (white bars), 20 mg/kg (black bars) and 40 mg/kg (grey bars). Figures represent mean ± SEM derived from 5-6 animals from different litters. #$p<0.05$ or less vs. control + 0 mg/kg AT; *$p<0.05$ vs. STZ + 0 mg/kg AT by a one-way ANOVA with a Newman-Keuls Multiple Comparison Test.


A
Day 14
Control
STZ
Total islets/pancreatic section (mm²)
AT Dose (mg/kg)
B
Day 44
Control
STZ
Total islets/pancreatic section (mm²)
AT Dose (mg/kg)
C
Control
STZ
Small islets/pancreatic section (mm²)
AT Dose (mg/kg)
D
Control
STZ
Small islets/pancreatic section (mm²)
AT Dose (mg/kg)
E
Control
STZ
Medium islets/pancreatic section (mm²)
AT Dose (mg/kg)
F
Control
STZ
Medium islets/pancreatic section (mm²)
AT Dose (mg/kg)
G
Control
STZ
Large islets/pancreatic section (mm²)
AT Dose (mg/kg)
H
Control
STZ
Large islets/pancreatic section (mm²)
AT Dose (mg/kg)

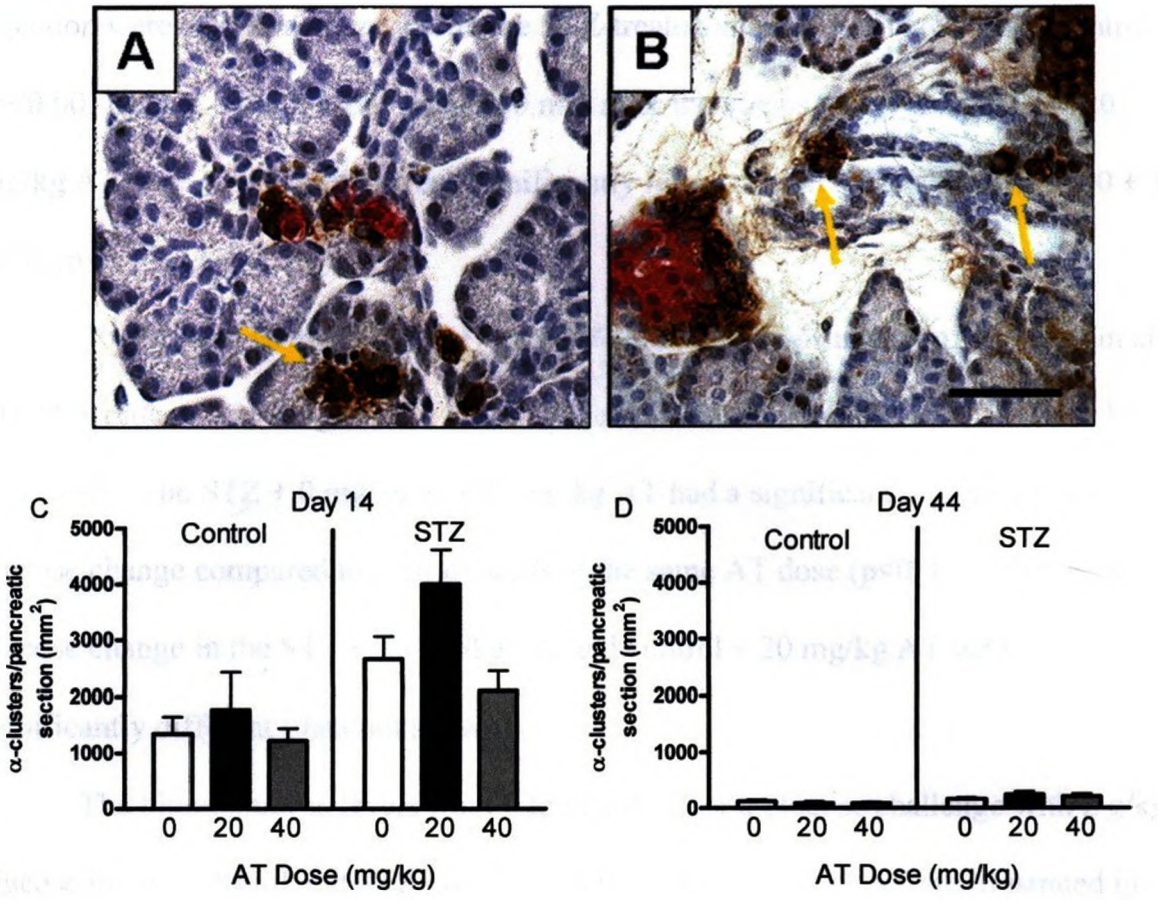


Figure 2.9. Micrographs depicting immunohistochemical localization of α-cell clusters (200-5000 μm^2) in the rat pancreas (A) and budding off ducts (B) from STZ + 20 mg/kg AT treated animals on PD 14. The α-cells (brown) are stained with anti-glucagon. *Yellow arrows* indicate the α-cell clusters (A-B). Scale bar=50 μm. α-cell cluster area at PD 14 (C) and PD 44 (D) in both control and STZ-treated animals, given a dose of 0, 20 or 40 mg/kg AT. Control and STZ-treated animals were analyzed separately. The AT dose was represented as follows in both the control and STZ groups; 0 mg/kg (white bars), 20 mg/kg (black bars) and 40 mg/kg (grey bars). Figures represent mean ± SEM derived from 5-6 animals from different litters.

injection were significantly higher in the STZ-treated animals compared to the controls ($p<0.001$). The blood glucose values 30 min after the glucose load in the STZ + 20 mg/kg AT dose (24±1.6 mM) were significantly lower than in STZ without AT (30 ± 1.3 mM) ($p<0.01$) (Table 2.3).

The percent change of blood glucose levels were measured from 0 to 30 min at PD 44 and there was a significant effect of the combined treatments of STZ and AT ($p<0.001$). The STZ + 0 mg/kg and 40 mg/kg AT had a significantly greater blood glucose change compared to their controls at the same AT dose ($p<0.01$). The blood glucose change in the STZ + 20 mg/kg AT and control + 20 mg/kg AT were not significantly different (data not shown).

The blood glucose levels from 0 to 90 min after a glucose challenge with 2 g/kg glucose in both control and STZ groups with increasing doses of AT are illustrated in Figure 2.10A. The STZ groups showed elevated blood glucose levels throughout the treatment compared to the control groups. The analysis of the area under the curve (AUC) showed a significant effect of the combined treatment of STZ and AT ($p<0.05$) (Figure 2.10B). The AUC for the STZ groups was significantly higher than the controls ($p<0.001$), although within the STZ treatment, the AUC for STZ + 20 mg/kg AT was significantly less than the STZ alone ($p<0.01$), indicating a better glucose tolerance in the STZ + 20 mg/kg AT animals (Figure 2.10B).

2.3.10 Pancreatic and serum insulin content

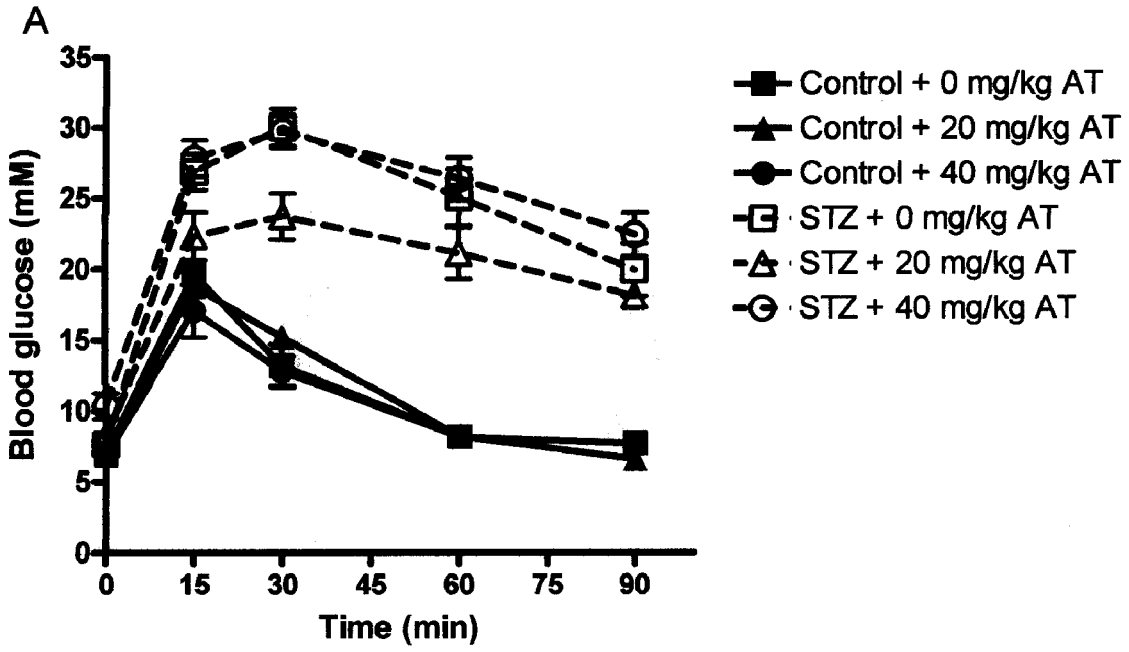
There were no differences in fasted serum insulin at PD 44 (Figure 2.11A). Furthermore, there were no differences in pancreatic insulin content between AT doses, within both the control and STZ-treated groups, although the STZ groups both with and

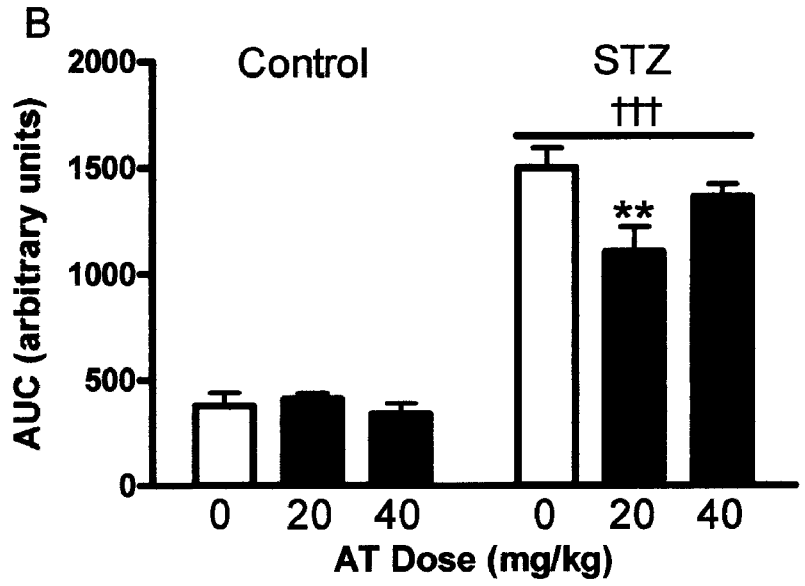
Table 2.3. Blood glucose was compared at 0 min (after a 5 h fast) and 30 min (following 2g/kg glucose ip) at PD 44. Control and STZ-treated animals, treated with an AT dose of 0, 20 or 40 mg/kg were compared.

			AT Dose (mg/kg)		
			0	20	40
Blood Glucose (mM)	0 min	Control	7.0±0.1	6.9±0.4	6.9±0.3
		STZ	7.6±0.6	8.2±0.3	10±0.9#
	30 min	Control	13±1.5	15±0.2	13±1.1
		STZ	30±1.3###	24±1.6###,**	30±1.2###

Data are mean ± SEM; n=4-8 animals from different litters/group. All 30 min time points have significantly greater blood glucose levels than the 0 min time point ($p<0.001$). ###$p<0.001$ vs. control of the same dose at 30 min, # $p<0.05$ vs. control of the same dose at 0 min; **$p<0.01$ vs. STZ + 0 mg/kg at 30 min by a two-way ANOVA with Bonferroni post test.

Figure 2.10. A glucose tolerance test was performed in control and STZ-treated rats with or without the addition of 20 or 40 mg/kg AT at PD 44 (A). Solid line (control), dashed line (STZ); square (0 mg/kg AT), triangle (20 mg/kg AT), circle (40 mg/kg AT). To determine differences in blood glucose levels, the AUC was determined for each group (B). Control and STZ-treated animals were compared. The AT dose was represented as follows in both the control and STZ groups; 0 mg/kg (white bars), 20 mg/kg (black bars) and 40 mg/kg (grey bars). There was a combined effect of the STZ treatment and AT dose ($p<0.05$). Figures represent mean ± SEM derived from 4-8 animals from different litters. †††$p<0.001$ vs. control of the same dose; **$p<0.01$ vs. STZ + 0 mg/kg AT by a two-way ANOVA with Bonferroni post test.


A
Blood glucose (mM)
35
30
25
20
15
10
5
0
0
15
30
45
60
75
90
Time (min)
Control + 0 mg/kg AT
Control + 20 mg/kg AT
Control + 40 mg/kg AT
STZ + 0 mg/kg AT
STZ + 20 mg/kg AT
STZ + 40 mg/kg AT


B
AUC (arbitrary units)
2000
1500
1000
500
0
Control
STZ
†††
**
0
20
40
0
20
40
AT Dose (mg/kg)

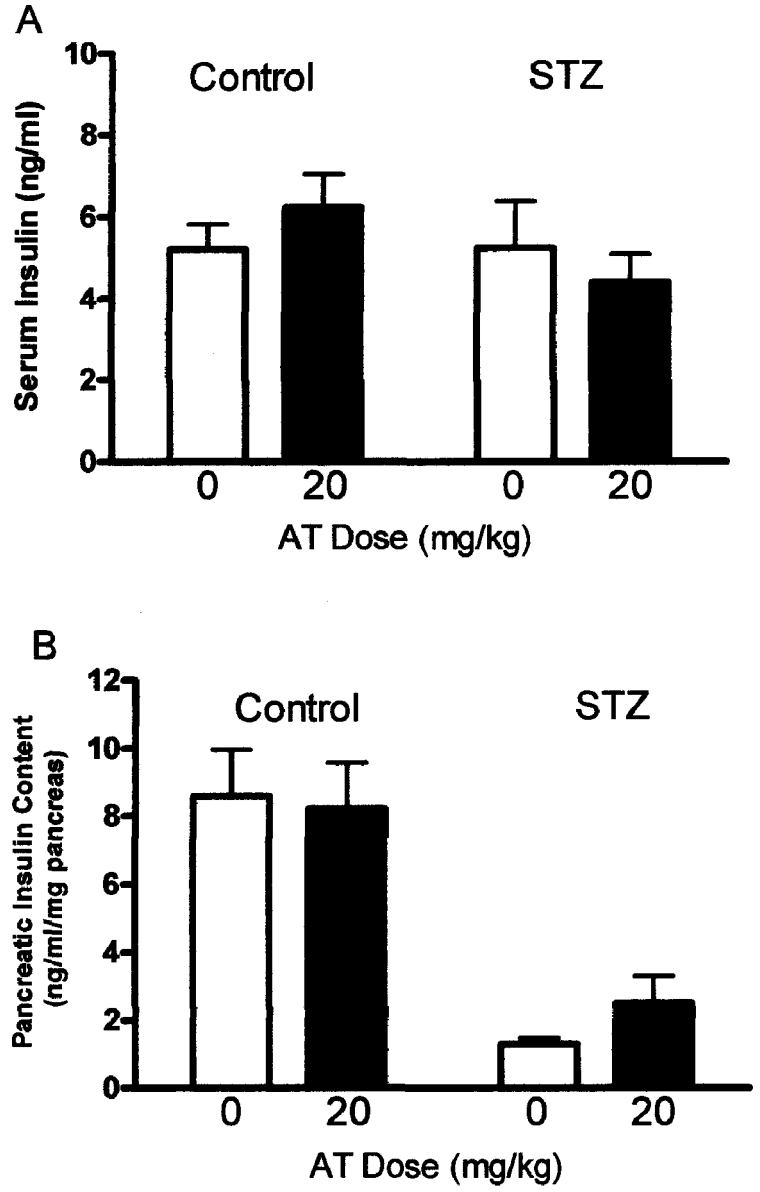


Figure 2.11. The serum insulin concentration (A) and the pancreatic insulin content (B) as determined by RIA at PD 44 (B). Values were normalized to pancreas weight (mg). Control and STZ-treated animals were compared. The AT dose was represented as follows in both the control and STZ groups; 0 mg/kg (white bars) and 20 mg/kg (black bars). Figures represent mean ± SEM derived from 8-10 animals from different litters. There was a significant effect of the STZ treatment ($p<0.001$) by a two-way ANOVA (B).

without AT had significantly lower pancreatic insulin content than controls ($p<0.001$) (Figure 2.11B).

2.3.11 Immunolocalization of BS-1 lectin and Flk1 in pancreas

The BS-1 staining used to visualize the pancreatic islet microvessels produces a dense background staining in the exocrine tissue so the vascular density cannot be evaluated there (22). The pancreata were morphometrically analyzed to identify the presence of BS-1 lectin and Flk1, and to examine changes in the quantity of ECs and their potential contribution to tissue growth and regeneration in the pancreatic islets.

BS-1 lectin was visualized as red cytoplasmic staining (Figure 2.12B), while Flk1 was visualized as grey membrane staining (Figure 2.12C). The flk-1 ECs and the dual positive BS-1 lectin/ Flk1 ECs were only seen at PD 6 and PD 14 (Figure 2.12D), while the BS-1 lectin-positive cells were seen at PD 6, PD 14 and PD 44. A negative control was prepared in order to determine antibody specificity (Figure 2.12A). At all three time points the majority of the cells were found in association with islet capillaries mainly in the central core of the islet.

There was a significant increase in the number of BS-1 lectin-positive cells at PD 6 in the STZ-treated animals ($p<0.05$) (Figure 2.13A) and there were very few Flk1-positive cells (Figure 2.14A) and some were located in the periphery of the islet. At PD 6, the BS-1 lectin/ Flk1 ECs were most abundant (Figure 2.14C).

The amount dual positive BS-1 lectin/Flk1 and Flk1-positive cells were larger at PD 14 (Figure 2.14B and D) than at PD 6 (Figure 2.14A and C). AT treatment was associated with the significant increase in the number of BS-1 lectin-positive cells ($p<0.05$) (Figure 2.13B) and BS-1 lectin/Flk1-positive cells ($p<0.01$) (Figure 2.14D) at PD 14.

By PD 44, there were no differences in the amount of BS-1 lectin-positive cells between groups (Figure 2.13C). The larger the islet, the more BS-1 lectin staining was observed indicating that size of the islet was correlated with the amount of ECs. There were no Flk1-positive or dual positive cells visible at PD 44.

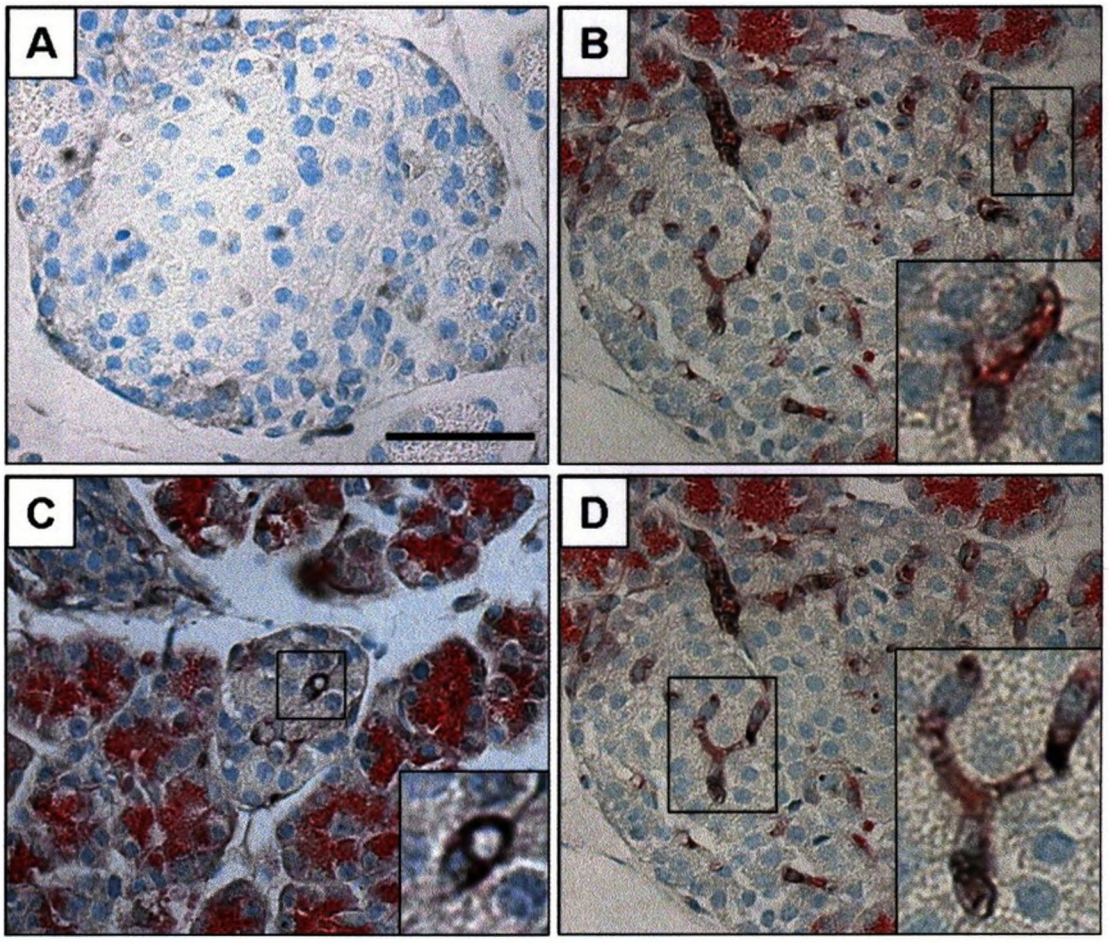
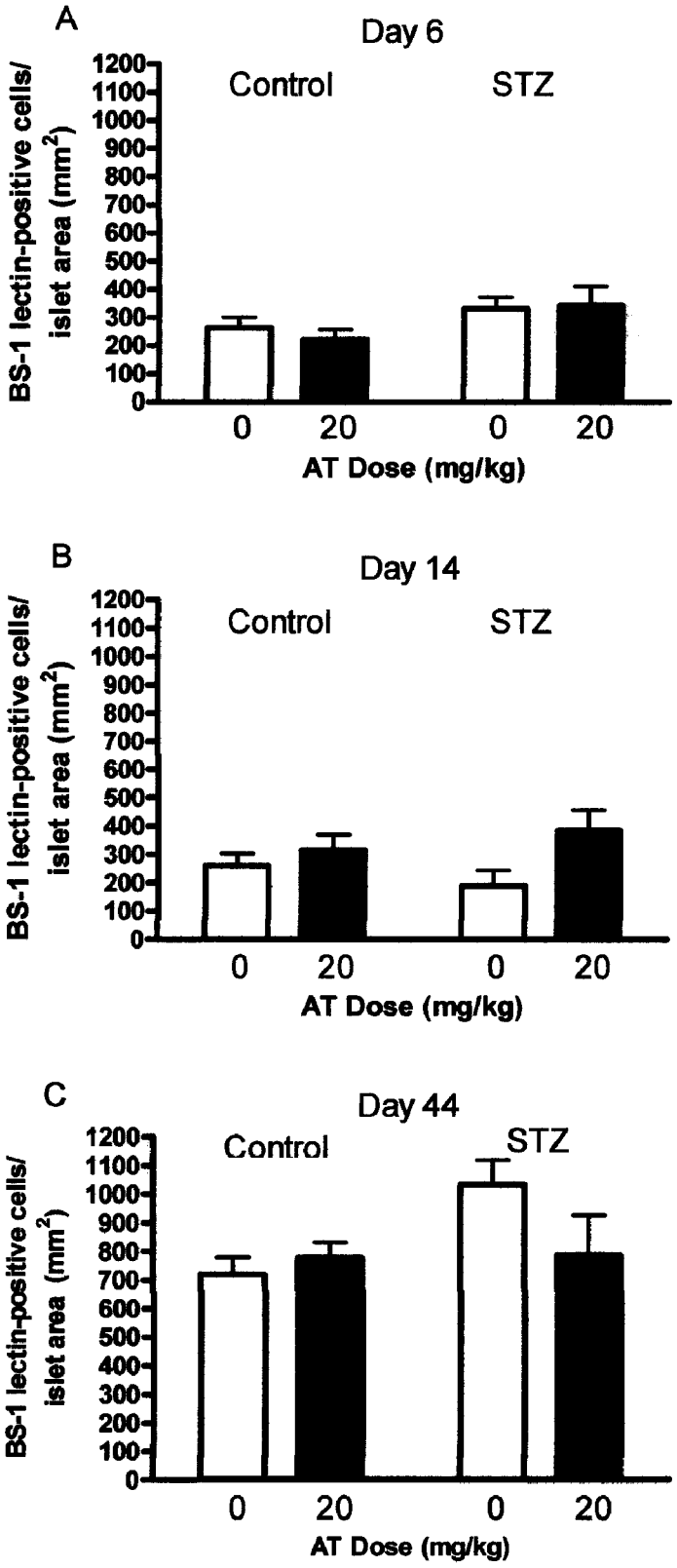


Figure 2.12. Micrographs showing localization of BS-1 lectin (red) and Flk1 (grey) in rat pancreata at PD 14. Negative controls were done with no primary antibodies (A). The islets in control (B and D) and STZ-treated (C) animals are shown. A BS-1 lectin-positive cell (B), Flk1-positive cell (C), and dual positive BS-1 lectin/Flk1 cell (D) are shown. *Boxes* are shown at a greater magnification at the bottom right. Scale bar=50 μm.

Figure 2.13. BS-1 lectin-positive cells per islet area at PD 6 (A), PD 14 (B), and PD 44 (C) in both control and STZ-treated animals, treated with a dose of 0 mg/kg AT (white bars) or 20 mg/kg AT (black bars). Control and STZ-treated animals were compared. Figures represent mean ± SEM derived from 5-6 animals from different litters. There was a significant effect of the STZ treatment ($p<0.05$) (A) and a significant effect of the AT dose ($p<0.05$) (B) by a two-way ANOVA.


A
Day 6
Control
STZ
BS-1 lectin-positive cells/ islet area (mm²)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0
20
0
20
AT Dose (mg/kg)
B
Day 14
Control
STZ
BS-1 lectin-positive cells/ islet area (mm²)
0
20
0
20
AT Dose (mg/kg)
C
Day 44
Control
STZ
BS-1 lectin-positive cells/ islet area (mm²)
0
20
0
20
AT Dose (mg/kg)

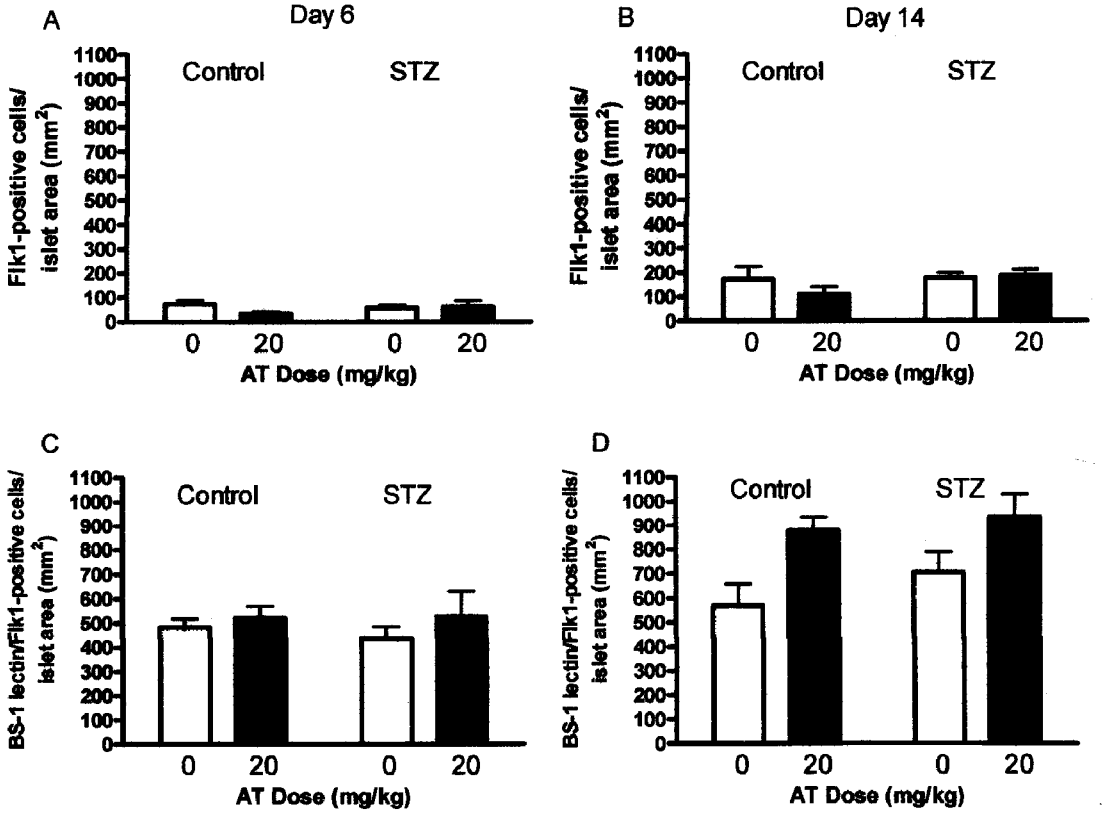


Figure 2.14. Flk1-positive cells per islet area at PD 6 (A) and PD 14 (B) and BS-1 lectin/Flk1-positive cells per islet area at PD 6 (C) and PD 14 (D) in both control and STZ-treated animals, treated with a dose of 0 mg/kg AT (white bars) or 20 mg/kg AT (black bars). Control and STZ-treated animals were compared. Figures represent mean ± SEM derived from 5-6 animals from different litters. There was a significant effect of the AT dose ($p<0.01$) (D) by a two-way ANOVA.

2.4 Discussion

The objective of this study was to examine the affect of AT on the growth and regeneration of β-cells, and to determine if this process involved neovascularization. We hypothesize that the rate and capacity of the β-cell growth and regenerative processes will be enhanced with an increasing AT dose, and that EPCs could be involved in neovascularization which will influence β-cell replacement.

We used an STZ model that was previously reported in our laboratory (10), and we primarily examined β-cell mass at PD 6 and found a 75% reduction in the STZ-treated animals. We also determined that the offspring had AT present in their plasma at birth. There was a significant increase in β-cell mass at PD 44 within both the control and STZ groups treated with AT. The 20 mg/kg dose had a similar outcome in both the control and STZ-treated groups, although there was a larger increase in β-cell mass in the STZ-treated group from PD 14 to PD 44 when compared to the controls.

There was a significant increase in total islets in the control animals treated with both AT doses, and the STZ treatment with the 20 mg/kg AT dose which were both correlated to the increase in the number of small islets. These findings illustrate that AT has a potential role in the mechanism of neogenesis in β-cell regeneration. The 20 mg/kg AT dose was stimulating neogenesis in the STZ-treated rodents, rather than hypertrophy of the β-cells, as we saw with the 40 mg/kg dose.

In our laboratory, we have found that after STZ treatment there was increased cell proliferation in both insulin- and glucagon-positive cells and also α-cell hyperplasia (10). It is not understood whether these α-cell clusters are the remnants of islets after STZ damage or primitive islets that have yet to develop into β-cells. We suggest that the

increase in α-cells we see at PD 14 may precede the later amplification of β-cells by PD 44 in the AT treated animals.

Due to the fact that we found an increase in the β-cell mass at PD 44, we examined the function of β-cells in these islets by subjecting the rats to an ipGTT. Control animals had similar responses to the glucose load regardless of the AT treatment. But, the STZ + 20 mg/kg AT-treated rats had an improved glucose tolerance compared to animals without AT or those receiving 40 mg/kg AT. There were no differences in fasted serum insulin levels in the islets of the STZ-treated animals at PD 44 compared to the controls, although the STZ groups both with and without AT had significantly lower pancreatic insulin content. This was most likely due to the fact that the new β-cells were immature and not able to deal with the increasing metabolic demands. Although there were less β-cells in the STZ-treated animals, the mature functioning β-cells compensated and secreted relatively more insulin.

EPCs do not have definitive markers in the rat, so we chose to examine the changes in ECs both within capillaries and present as individual cells using BS-1 lectin binding and Flk1 expression. BS-1 lectin has been used previously to detect endothelium within the islets (22), and Flk1, one of the earliest differentiation markers for ECs (24;25), is expressed on the ECs within the islet and is known to promote angiogenesis (26;27). Examining these cell markers allowed us to determine if our treatments likely promoted angiogenesis.

In the STZ-treated animals at PD 6, the number of BS-1 lectin-positive cells was larger when compared to the controls indicating more ECs in these animals. Treatment with AT was associated with an increase in the number of BS-1 lectin-positive ECs and dual positive BS-1 lectin/Flk1 ECs at PD 14, but not at PD 44. This could be an

indication that the effect of AT on angiogenesis had diminished after the treatment was stopped at PD 14.

Quiescent vascular ECs are activated by proangiogenic growth factors which regulate/stimulate EPC and EC proliferation, migration, and differentiation, thereby promoting new vessel formation (19;28;29). Previously, endothelial regeneration processes were thought to involve proliferation and migration from pre-existing mature ECs adjacent to the site of injury. However, circulating bone marrow derived EPCs were shown to home in on sites of endothelial disruption and become incorporated into the nascent endothelium (30). The discovery of EPCs led to the new concept that vasculogenesis and angiogenesis occur simultaneously in postnatal life because these cells were able to differentiate into vascular endothelium when needed, through a mechanism recapitulating embryonic vasculogenesis. EPCs have the capacity to proliferate, migrate, and differentiate into endothelial lineage cells, but have not yet acquired the characteristics of mature ECs (31).

The increase in the β-cell mass could potentially be due to EPC mobilization to the pancreas. Statins have the ability to upregulate and mobilize EPCs which have been shown to localize to the injured pancreas (32). Intra-islet ECs and mobilized EPCs may contribute to regeneration of β-cells by incorporating into sites of neovascularization and enhance blood vessel formation, which in turn will stimulate and enhance growth signals. Collectively, these findings suggest that a substantial plasticity exists in the endocrine pancreas, and that the regenerative capacity could be due to inductive signals.

It is evident that tissue injury is a potent stimulus for neovascularization and it is plausible that strategies that further enhance pancreatic neovascularization could lead to β-cell neogenesis, proliferation, or improved function and this might be achieved through

optimization of the AT dose, timing or route of EPC delivery and/or coadministration of growth factors such as VEGF which stimulate the proliferation and/or localization of ECs (33;34).

In conclusion, our findings support our hypothesis that β-cell mass will be expanded with AT and we observed a better glucose tolerance in STZ animals treated with 20 mg/kg AT. Treatment with AT following pancreatic injury appears to affect the neogenesis of islets in control animals and enhances the regeneration process in STZ-treated animals. Our findings suggest a novel effect of statins and their role in regeneration and will help further elucidate the mechanisms of β-cell growth and regeneration.

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CHAPTER 3

GENERAL DISCUSSION

3.1 Study objective

The objective of this study was to examine the affect of AT on the growth and regeneration of β-cells, and to determine if this involved neovascularization. Our rationale was that the mobilization of EPCs could possibly induce β-cell mass expansion by inducing neovascularization, which would increase the appropriate paracrine signals, such as growth factors or cytokines, leading to β-cell proliferation, differentiation and neogenesis. No prior studies have looked at the pleiotropic effects of AT on the morphogenesis of the pancreas, with or without STZ injury. Primarily, we wanted to determine if there was an enhancement in the regeneration of the β-cell mass, changes in distribution of islets within the pancreas, improved glucose tolerance and if there was an increase in islet ECs.

3.2 Biological significance

3.2.1 The effect of STZ on β-cell mass

In order to determine that STZ induced β-cell loss as previously reported (1), we examined the β-cell mass at PD 6 and found a 75% reduction in the STZ-treated animals. As well, there was histological evidence of new islet formation at PD 14. By PD 44, no evidence of remaining damage was apparent in the islets. Although, the STZ animals had recovered some of their β-cell mass, this was only half of the control values by PD 44.

3.2.2 The effect of AT on the offspring during gestation and early neonatal life

In order to determine that the AT administered to the pregnant rats was crossing the placenta, the parent compound of AT was measured in pups at PD 1 (birth) and we determined that in the offspring whose mother's had received AT, AT was present in their

plasma. At PD 6, the pups were likely only receiving the metabolites of AT from the mother through lactation, therefore we were unable to detect any parent AT, although some of these AT metabolites are known to be as active as the parent compound (2).

A previous study by Henck et al. (3), showed that a single dose of 10 mg/kg AT given to female rats on GD 19 or PD 13 showed evidence of placental transfer and excretion into the milk. This was analyzed using radiolabelled ^{14}C-AT which was detected in the fetal circulation and liver 6 h following oral administration to female rats on GD 19, indicating that AT and/or metabolites underwent placental transfer (3). The concentration of ^{14}C-AT in the fetal liver was 0.176 μg/g, while in the entire foetus it was 0.035 μg/g, suggesting hepatic extraction of radioactivity. Pregnant rats also had high liver radioactivity concentrations with a mean of 26.7 μg/g, relative to mean placental and plasma values of 0.111 μg/g and 0.178 μg/ml, respectively (3). This suggests that approximately 3% of the radioactively labelled-AT in the placenta (0.111 μg/g) is passed to the foetus (0.035 μg/g).

Furthermore, ^{14}C radioactivity was detected in nursing pups 6 h following oral administration of ^{14}C-AT to lactating female rats on PD 13, indicating AT metabolites undergo excretion into the milk. Lactating rats whose pups were removed 3 h prior to milk collection had a mean concentration of ^{14}C-AT in the plasma and milk at 0.099 and 0.102 μg/ml, respectively. Nursing pups that remained with the dam through 6 h post-dose, had mean plasma and liver concentrations of 0.050 μg/ml and 0.037 μg/g, while dams of these pups had had mean plasma and liver concentrations of 0.077 μg/ml and 10.7 μg/g (3).

Age and weight-matched females had the maximum plasma concentration (C_{max}) of AT at 60 min post-gavage. This was similar to previous findings by Dostal and Henck,

who found that the t_{max} and $t_{1/2}$ values in females ranged from 1 to 5 h and 2.5 to 5 h using doses of 20, 100 and 225 mg/kg. The C_{max} in females were approximately 80 to 100 ng/ml with the 20 mg/kg dose (3;4), which was similar to our findings using the same dose. In these experiments, the mean C_{max} increased in a greater than proportional manner with increasing dose, but t_{max} and $t_{1/2}$ did not change as a function of the dose (4). It was not clear whether AT pharmacokinetics were dose-proportional in rats.

We found no adverse effects of AT administration in pregnant mothers or their offspring at either a dose of 20 mg/kg or 40 mg/kg AT when we examined body weight and litter size. Previous studies from Henck et al. (3) indicated that maternal toxicity occurred at a 225 mg/kg/day dose given from GD 7 through PD 20. Developmental toxicity, which was characterized by increased preweaning mortality, reduced offspring body weight, developmental delay, and behavioural changes were observed with the administration of 225 mg/kg of AT, and reduced offspring body weight and behavioural changes also occurred at 100 mg/kg, a dose which did not result in adverse effects on the mother (3;4). A 20 mg/kg/day dose had no adverse effects on the offspring (3).

In the STZ-treated animals, non-fasting blood glucose levels were higher at PD 6 and this confirmed that the β-cells were damaged. The blood glucose values and the reduction of β-cell mass were similar in animals treated with and without AT indicating no protective effect of AT in preventing β-cell damage when STZ was administered.

3.2.3 An increase in β-cell mass with AT treatment

There was a significant increase in β-cell mass at PD 44 with AT within both the control and STZ treatment groups, indicating increased growth in the control animals and enhanced regeneration in the STZ-treated animals. The 20 mg/kg dose had a positive

effect on β-cell growth in both the control and STZ treated groups, while the 40 mg/kg dose had a negative effect on β-cell growth in the STZ group at PD 14.

The 20 mg/kg dose showed a similar β-cell increase in both the control and STZ treated groups, although there was a larger increase in β-cell mass in the STZ treated group from PD 14 to PD 44 when compared to the controls. At PD 14, the addition of AT did not have a significant effect on the regeneration of the β-cell mass, but during the period between PD 14 and PD 44, AT had an effect on the rate at which the β-cell mass increased.

3.2.4 Neogenesis of islets with the addition of AT

The changes in islet distribution at PD 44 in the control and STZ treatment were similar. The significant increase in total islets seen in the control animals treated with both AT doses, and following STZ treatment with the 20 mg/kg AT dose, could be accounted for by the increase in the number of small islets.

The findings illustrate that AT has a potential role in the mechanism of neogenesis in β-cell regeneration and suggest that the 20 mg/kg AT dose was stimulatory to neogenesis in the STZ-treated rats, rather than causing hypertrophy of the β-cells, as was seen with the 40 mg/kg dose. Islet neogenesis has been reported previously after STZ treatment (1;5;6). In a previous study in our laboratory using STZ, the loss of β-cells was rapidly followed by the increased number of individual insulin- and glucagon-positive cells associated with the pancreatic ducts, and an increase in the proliferative activity of the ductal epithelium (1). Within 8 d of STZ treatment (PD 12), numerous small insulin-positive cell clusters were seen adjacent to the pancreatic ducts. Within pre-existing islets there was increased cell proliferation in both insulin- and glucagon-positive cells and also α-cell hyperplasia (1). These changes occurred in our experiment and were evident by

PD 44. We observed that at PD 14, many α-cell clusters were present within the pancreas, as well as budding off the ducts. These α-cell clusters were more frequent at PD 14 when compared to PD 44. Another study found that around PD 20 in the developing pancreas of Wistar rats, there was a high incidence of small groups of α-cells (7). Therefore, α-cells may have also contributed to the increase in β-cell mass through inter-islet signalling.

It is not understood whether these clusters are the remnants of islets after STZ damage or primitive islets that have yet to develop into β-cells. We suggest that the increase in α-cells precede the later amplification of β-cells by PD 44 in the AT treated animals. In a previous study using the same STZ model, we found that these α-cells were co-localized with either Pdx1 or Glut2 expression, suggesting that they may be an immature β-cell phenotype (1). This increase in α-cells likely caused an increase in the processing of GLP-1. While this suggests that GLP-1 has a role in the regenerative pancreatic micro-environment, the target cells for GLP-1 action are still unclear. However, the administration of GLP-1 for 10 d to neonatal diabetic rats following partial pancreatectomy stimulated expansion of β-cell mass via the induction of islet proliferation and islet neogenesis (8).

Some authors imply that the capacity to regenerate following a toxic insult like STZ rapidly declined during the first 5 days of life in rats (9). Recent studies by Brand et al. (10) and Rosenberg et al. (11) indicate that in the STZ rat model, islet neogenesis is a significant contributor to β-cell mass expansion. In contrast, Butler et al. (12) have shown that β-cell mass expansion occurs primarily via proliferation in the mouse while in human subjects, β-cell neogenesis appears to be upregulated while β-cell replication is

unchanged (13). A controversy still remains as to whether β-cell hyperplasia or neogenesis is the major contributor to expansion (14).

β-cell neogenesis may occur by stem/progenitor cell activation (14). This refers to the differentiation of an inactive cell expressing primordial markers to yield insulin producing β-cells. Whether or not such a pancreatic stem cell exists is still controversial (15;16). Theories suggest the possibility of a multipotent stem cell population or the ability of differentiated endocrine cells to de-differentiate into a precursor cell phenotype, that will then become capable of expansion and re-differentiation along the β-cell lineage (14;17). The presence of organ-specific stem cells or precursors of the endocrine pancreatic lineages within the pancreatic ducts has been proposed, but no cell type of specific phenotype has been isolated (17).

Experimental models in rodents have shown that β-cells can regenerate after tissue injury, apparently through differentiation of precursor cells located within the pancreatic ducts (18;19). There has been clear evidence that a scarce subpopulation of multipotent precursor islet precursor cells resident in both ducts and islets of the adult mouse pancreas (16). In our experiment, AT was stimulating regeneration at PD 44 in the STZ animals, as we saw many new small islets, which was suggestive of regeneration. The signals or precursor cells would appear to be active at least this long.

The β-cell mass is dynamic and varies according to the need for insulin secretion (20;21). Increased insulin requirements promote β-cell replication and neogenesis, leading to an increased islet size and number. As islet mass increases, angiogenesis increases the capillary bud to provide oxygen and nutrients to new endocrine cells, preserves islet architecture, and may influence apoptosis (20).

3.2.5 Islet functionality with the 20 mg/kg AT dose

Due to the fact that we found an increase in the β-cell mass at PD 44, we examined the functionality of those islets by subjecting the rats to an intraperitoneal glucose tolerance test (ipGTT). The control animals had similar responses to the glucose load regardless of the AT treatment. But, in the STZ + 20 mg/kg AT treated rats there was an improved glucose tolerance compared to no AT or those receiving 40 mg/kg AT. Fasting blood glucose levels in the STZ + 40 mg/kg AT were significantly higher than animals treated without AT and 20 mg/kg AT, suggesting that this dose was detrimental to β-cell function.

Previous studies have shown that foetal and neonatal β-cells display immature functional features characterized by diminished sensitivity to glucose, limited insulin secretion and the absence of a biphasic response to glucose (22-24). In contrast, adult β-cells show a robust, biphasic insulin secretion (25;26) and consistently respond to different glucose concentrations by secreting incremental amounts of insulin. In our studies, the new, small islets in the STZ-treated animals were most likely immature, but with the addition of 20 mg/kg AT dose, the β-cell function and possible maturation, was increased and they were able to handle the glucose challenge more effectively.

In islets of the STZ-treated animals at PD 44, we found no differences in fasted serum insulin values compared to the controls, although the STZ groups both with and without AT had significantly lower pancreatic insulin content. This was most likely due to the fact that the new, small β-cells were immature and not able to deal with the increasing metabolic demands. Although there were less β-cells in the STZ-treated animals, the mature functioning β-cells compensated and secreted relatively more insulin.

In previous studies, we found that pancreatic insulin messenger ribonucleic acid (mRNA) was substantially lower in STZ-treated rats on PD 16 and had not recovered until PD 40, suggesting that the recovered β-cell mass at PD 16 was not functionally mature (1). It has been shown that after STZ-induced injury the surviving β-cells were able to maintain most of their metabolic functions but failed to maintain an adequate insulin production (27).

In a study by Satoh et al. (28), the oral glucose tolerance test (oGTT)-induced insulin secretion in adult Wistar rats appeared to be enhanced by statin treatment for more than 6 wk, as compared to Wistar control rats. The dosage of AT at 8 mg/kg/day in this experiment was about 12 to 24 times higher than the respective clinical usage (28). Another study by Suzuki et al. (29), used an oral 30 mg/kg dose of AT for 4 wk on the KK/Ay mice, an animal model of obesity-associated type 2 diabetes with insulin resistance. After administering an oGTT (20% glucose solution 2g/kg), plasma glucose levels were significantly lower at 30, 60 and 120 min in the AT-treated animals. Plasma insulin levels were also lowered before the GTT, and 30 and 60 min after glucose loading. They determined that AT may improve insulin resistance (29).

Conversely, in a study by Kanda et al. (30), they reported a moderately worse oGTT (5 g/kg glucose solution) in STZ-induced rats (24 mg/kg intravenous (iv)) treated with an oral dose of 8 mg/kg AT for 6 wk. A study by Palomer et al. (31) used two models of type 1 diabetes; the spontaneous non-obese diabetic (NOD) and the multiple low-dose (60 mg/kg ip for 5 consecutive days) STZ mice. They used AT doses of 10 mg/kg dietary AT and 20 mg/kg ip injected AT for 30 d, and found no significant effect on reducing or delaying the onset of diabetes in both the NOD or STZ mice (31).

The lack of effect in both studies could be explained by the low dose of AT used or the administration route. Some authors argue that the pleiotropic effects of statins in mice may require a dose up to 40mg/kg/day. Although these doses are much higher than that used in humans, they may be necessary in murine studies because of the rapid upregulation of HMG-CoA reductase in response to treatment (32). These studies differed from ours as we used a higher dose of STZ (70 mg/kg ip) and therefore destroyed more β-cells. Our AT dose was 20 or 40 mg/kg while other studies tend to use lower doses or different routes of administration. The animal models used were also different and this is important as species differences do exist between human and rodent pancreas with respect to cellular composition, tissue organization, and β-cell replicative activity (21). The animals in the previous studies cited were 4 to 8 wk old, which may have limited the regenerative capacity of the pancreas compared to younger animals. Furthermore, none of these studies determined the concentration of AT in the blood.

3.2.6 Effects of the high AT dose on pancreatic regeneration

We also examined the effects of a high dose of 40 mg/kg AT on the pancreas and body weight. The pancreas weight was increased at PD 44 in the animals that received the 40 mg/kg AT dose in both the control and STZ treatments. This dose of AT combined with STZ caused a decrease in β-cell mass at PD 14. Fasting blood glucose levels were significantly higher in the STZ-treated groups indicating moderate hyperglycaemia and the addition of AT did not change blood glucose levels at PD 14.

The 40 mg/kg AT dose in the STZ group at PD 44 showed an increased β-cell mass. To determine how the β-cell mass was increasing, the change in the size (hypertrophy) of β-cells was analyzed. When the individual β-cell size was examined, we found an increase in individual β-cell size in the control animals at PD 14 and the STZ

animals at PD 44. The β-cell hypertrophy seen at PD 44 in the STZ-treated animals could have contributed to the increased β-cell mass seen at this time. On the other hand, with the lower dose of 20 mg/kg, the changes in β-cell mass were likely due to an increased number of β-cells (hyperplasia). Neither dose affected the size of the exocrine cells at either PD 14 or PD 44.

These results suggest that the 40 mg/kg AT dose had an adverse effect on the pancreas causing a decrease in β-cell mass at PD 14 and then cell/organ hypertrophy at PD 44, thus indicating that when STZ treatment is combined with the 40 mg/kg AT dose, the effect was potentially harmful.

3.2.7 Islet ECs could contribute to increased β-cell mass

Due to the fact that EPCs do not have definitive experimental markers, we chose to examine the changes in islet ECs using BS-1 lectin binding and Flk1 expression. BS-1 lectin has been used previously to detect endothelium within the islets (33), and Flk1, one of the earliest differentiation markers for ECs (34;35), is expressed on the ECs within the islet and is known to promote angiogenesis (36;37). Examining these cell markers allowed us to determine if our treatments likely promoted angiogenesis.

In the STZ-treated animals at PD 6, the number of BS-1 lectin-positive cells was larger when compared to the controls indicating more ECs in these animals. Treatment with AT was associated with an increase in the number of BS-1 lectin-positive ECs and dual positive BS-1 lectin/Flk1 ECs at PD 14, but not at PD 44. This could be an indication that the effect of AT on angiogenesis had diminished after the treatment was stopped at PD 14.

Recent studies have suggested multiple interactions between islet ECs and β-cells. Many of these studies show a close correlation between increased islet vascularization

and islet mass. The pronounced growth of islet endocrine cells seen during the first week after birth coincides with an even more pronounced proliferation of islet ECs, which results in a marked increase in intra-islet vascular density (38). The islet capillary network undergoes considerable remodelling in the early postnatal period, which coincides with a rapid increase in islet mass after birth (39).

Proliferating endocrine cells are located close to islet ECs, suggesting an important role of blood vessels in islet growth. The growth factors involved in the simulation of β-cell proliferation may be produced locally by stromal cells or ECs (40;41). Studies have found that the β-cell specific deletion of VEGFA resulted in decreased islet vascular density (42). Another likely candidate, HGF, is a potent mitogen for both β-cells and ECs (43).

3.2.8 EPCs could contribute to neovascularization after injury

Quiescent vascular ECs are activated by proangiogenic growth factors which regulate/stimulate EPC and EC proliferation, migration, and differentiation, thereby promoting new vessel formation (44-46). Previously, endothelial regeneration processes were thought to involve proliferation and migration from pre-existing mature ECs adjacent to the site of injury. However, circulating bone marrow-derived EPCs were shown to home in on sites of endothelial disruption and become incorporated into the nascent endothelium (47). The discovery of EPCs led to the new concept that vasculogenesis and angiogenesis occur simultaneously in postnatal life because these cells were able to differentiate into vascular endothelium when needed, through a mechanism recapitulating embryonic vasculogenesis. EPCs have the capacity to proliferate, migrate, and differentiate into endothelial lineage cells, but have not yet acquired the characteristics of mature ECs (48).

EPC recruitment to sites of neoangiogenesis is triggered by the increased availability of angiogenic growth factors or chemokines (49). Once at the site of neovascularization, an EPC may recruit additional EPCs by releasing growth factors, such as VEGF or HGF (50). Organ regeneration results in the release of chemokines that recruit EPCs to the neoangiogenic site (51). Signalling from the vascular cells has been shown to be a critically important event in early pancreas development (52). Vascular ECs are proposed to secrete inductive factors necessary for the differentiation of stem/progenitor cells into the pancreatic lineage of development (53). Considering the relationship of endocrine and vascular cells within mature pancreatic islets, it seems plausible that the same signals seen in early pancreatic development would play a role.

Statins share certain activities with VEGF including the potential to promote neovascularization (54-56) and EPC mobilization in animals (55;57) and human subjects (58). Early neonatal life represents a period of accelerated tissue growth and active neovascularization and may provide a distinct milieu for vessel growth and formation and this is an important component of an adaptive response to β-cell injury (59). Several animal models have provided evidence that bone marrow-derived EPCs can contribute to neovascularization (60-62). Mobilization or transplantation of EPCs can enhance neovascularization and is associated with tissue regeneration and functional improvement after injury (55;63;64).

A study by Mathews et al. (59) in which C57BL/6J mice were treated with STZ and received transplanted bone marrow cells labelled with enhanced green fluorescent protein (EGFP), found evidence that bone marrow-derived cells were recruited to the pancreas in response to islet injury and over time more cells appeared in the area surrounding the islets and ducts. A significant proportion of the cells had the morphology

of ECs, localized to small blood vessels and expressed the endothelial marker Vwf. Transplantation without prior β-cell injury resulted in fewer donor-derived cells in the pancreas (59). They also found that the cells in the bone marrow that give rise to the ECs in the injured pancreas are located in the fraction of cells that contained the haematopoietic stem/progenitor cells.

Tissue injury is a potent stimulus for neovascularization. Bone marrow-derived EPCs have been shown to contribute to neovascularization in several models including retinal, myocardial and hind limb ischaemia (63;65;66). Neovascularization of the pancreas could be an important component of an adaptive response to β-cell injury if EPCs differentiate into ECs and promote neovascularization (59;67). Forming new blood vessels or increasing the density of pre-existing vessels may increase cell/tissue exposure to certain growth factors or serve to increase the metabolic sustenance, which may facilitate the β-cell regeneration process (52).

3.2.9 EPCs could contribute to tissue regeneration

Recent studies have suggested that tissue regeneration can occur upon the introduction of bone-marrow derived stem cells that have multiple effects, including the vascularization of damaged tissue (51). Vascularization may be facilitated by angiogenic factors released from ECs originating from transplanted bone-marrow stem cells (51;68). It is interesting to note that in many of the studies to date, the animals were adult mice or rats from 4 to 10 wk old, while in our study, we used animals in the neonatal stage of development when the regenerative capacity of the pancreas is greatest.

Treatment with 20 mg/kg simvastatin for 4 wk increased circulating EPCs in mice, and statin supplementation in culture medium increased proliferation, differentiation and function, while reducing senescence of EPCs (46;69). Furthermore,

treatment with 40 mg of AT for 4 wk in patients with CAD, increased and stimulated EPCs and improved differentiation into cardiomyogenic cells (70;71).

Hess et al. (72) reported that transplantation of bone marrow-derived stem cells initiated endogenous pancreatic regeneration in adult mice where the natural regenerative capacity is low. This resulted in the modest improvement in hyperglycaemia and elevated levels of serum insulin, thereby improving the metabolic state of the recipient animals. In these STZ-treated adult mice, there was an increase in the number of β-cells at both ductal and islet sites after bone marrow transplantation, but in animals without transplantation, there was no evidence of proliferation in ductal or islet structures (72). They also examined the pancreata of transplanted mice for ECs and found increased numbers of donor-derived ECs in STZ-treated animals, whereas non-STZ-treated transplanted mice had none, suggesting that pancreatic injury was required for the recruitment of bone-marrow donor ECs. This could involve bone-marrow derived EPCs, as up to 9% of donor cells in the pancreas stained positive for the vascular marker PECAM1 (72).

3.2.10 The potential role of EPCs in β-cell regeneration

Statins have the ability to upregulate and mobilize EPCs which have been shown to localize to the injured pancreas (59). Although we were not able to directly measure EPCs, we postulate that the observed increase in the β-cell mass could be an effect of EPC mobilization to the pancreas. It is possible that intra-islet ECs and mobilized EPCs may contribute to regeneration of β-cells by incorporating into sites of neovascularization and enhance blood vessel formation, which in turn will stimulate and enhance growth signals. Collectively, these findings suggest that substantial plasticity exists in the endocrine pancreas, and that the regenerative capacity is potentially due to inductive signals. It is evident that tissue injury is a potent stimulus for neovascularization and it is

plausible that strategies that further enhance pancreatic neovascularization could lead to β-cell neogenesis, proliferation, and improved function. This might be achieved through optimization of the AT dose, timing or route of EPC delivery and/or coadministration of growth factors such as VEGF which stimulate the proliferation and/or localization of ECs (49;73).

Taken altogether, it seems likely that EPCs could be important mediators of β-cell renewal in response to β-cell injury induced by STZ, and that mobilization of EPCs to the damaged pancreas is potentiated by AT (Figure 3.1). In most organs, tissue damage results in a regenerative response. But in some tissues like the pancreas, this regenerative capacity is limited with age. β-cell injury appears to be a potent stimulus for the recruitment of EPCs (59). Therefore, the interaction of the pancreas and vasculature could aid in the stimulation of β-cell regeneration through EPCs, which can readily home to sites of injury and contribute to new blood vessel formation in order to deliver the appropriate signals for tissue regeneration (59).

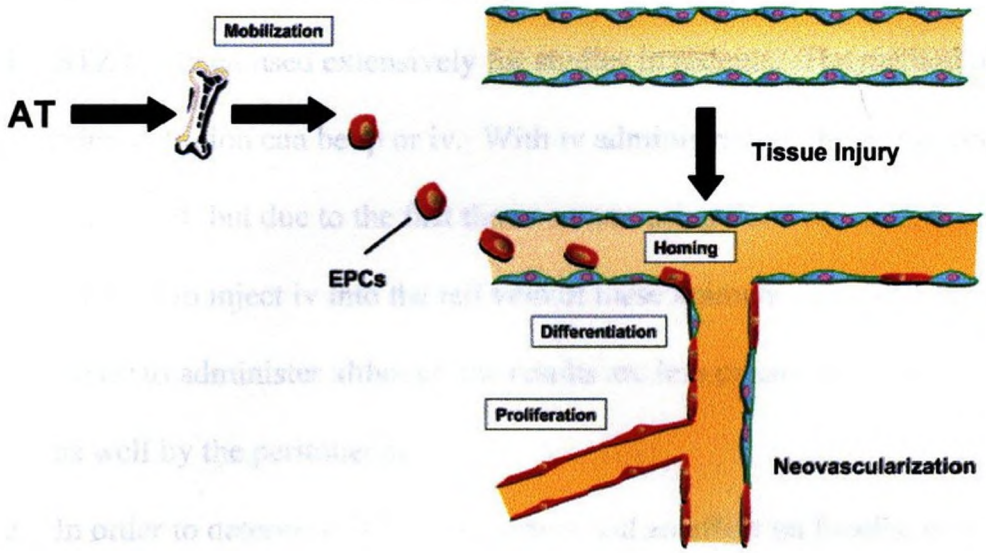

Figure 3.1. AT augments the EPC population and causes mobilization into peripheral blood where EPCs receive signals to home to sites of injury and contribute to neovascularization after STZ-induced injury in the neonatal rodent (Adapted from 74).

3.3 Limitations of the current study

1. STZ has been used extensively for studies in rodents. The method of administration can be ip or iv. With iv administration, the results are more consistent, but due to the fact that we were using PD 4 animals, it was extremely difficult to inject iv into the tail vein of these animals. The alternative, ip, is much easier to administer although the results are less consistent since it is not absorbed as well by the peritoneum.
2. In order to determine if STZ treatment had an effect on β-cells, non-fasting blood glucose values were tested at PD 6. We chose to do random, non-fasting blood glucose readings as the pups were lactating and to take them away from their mother's for an extended period of time at that age could be harmful. As well, with any blood glucose reading, stress often triggered an increase in the blood glucose levels.
3. As we wanted to know the effects of AT treatment on pancreatic development we decided to gavage the dams. The results obtained were therefore indirect as the pups received AT through either transplacental passage or via suckling.
4. We were unable to measure the amount of AT metabolites in the blood and milk of the pups, due to the unavailability of the metabolites to use as internal standards for HPLC/MS.

3.4 Recommendations/future studies

1. In future studies it would be important to investigate changes in the number and mobilization of EPCs specifically after AT treatment and to determine if they directly contributed to the increase in β-cell mass we observed.

2. In our studies, we pooled the data and did not study the differences in the metabolism of the drug between male and female pups. In future studies it would be interesting to determine any differences due to gender.
3. It appears that statins and VEGF have similar mechanisms by which they mobilize EPCs. It would be interesting to qualify and quantify growth factor changes, such as the levels of VEGF, and any other potential signals that could contribute to the β-cell mass expansion observed in this study.
4. It would also be important to study the changes in islet proliferation and determine the rate of proliferation in this model.
5. In order to determine if the effects of AT could be seen in older animals, similar experiments could be performed in juvenile and adult rats.
6. Finally, it would be important to characterize the signalling mechanisms that are involved in the interaction between EPCs, ECs and endocrine cells after AT treatment and STZ-induced β-cell injury.

3.5 Overall conclusion

In conclusion, our findings support our hypothesis that β-cell mass will be expanded following treatment with AT and we observed an improved glucose tolerance in STZ animals treated with 20 mg/kg AT. Treatment with AT following pancreatic injury appears to affect the neogenesis of islets in both control and STZ-treated animals. We also observed an increase in islet ECs which may have contributed to our observed increases in β-cell mass.

It is thought that the most active period of β-cell replication and neogenesis, which occurs early in life, will dictate the baseline for β-cell mass for the remainder of life (75). Our findings suggest a novel effect of statins and their role in regeneration and will help

further elucidate the mechanisms of β-cell growth and regeneration. Thus we will be one step closer towards finding a possible alternative treatment for diabetes mellitus. *In vivo* induction of pancreatic regeneration and expanding the endogenous β-cell mass, would eliminate the necessity for donor tissue and enable the application of regeneration therapy to restore normoglycemia (14).

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APPENDICES

Appendix 1. All animal procedures were performed with the approval of the Animal Care Committee of the University of Western Ontario in accordance with the guidelines provided by the Canadian Council for Animal Care.

December 15, 2004

This is the 2nd Renewal of this protocol
* A Full Protocol submission will be required in 2006*

Dear Dr. Hill:

Your "Application to Use Animals for Research or Teaching" entitled:

"Pancreatic Beta Cell Renewal in Development: Implications in Diabetes"
Funding Agency– CIHR Grant # R0362A04

has been approved by the University Council on Animal Care. This approval is valid from January 1, 2005 to December 31, 2005. The number for this project remains as #2002-114 -12 .

1. *This number must be indicated when ordering animals for this project.*
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED					FOR 1 YR	PAIN LEVEL - C
Rats	- Wistar	200 gm	F	-	60	
	- Wistar	200 gm	M	-	10	
Mice	- FVB/N (transgenic)	Preg/litter	M/F	-	30	
	- FVB/N (transgenic)	adult	M	-	15	
	- FVB/N (wild type)	Preg/litter	M/F	-	30	
	- FVB/N (wild type)	adult	M	-	15	

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (kfloyd@uwo.ca) in case of difficulties or if you require copies.
SOP's are also available at http://www.uwo.ca/animal/acvs
310 Holding Period Post Admission
320 Euthanasia
321 Criteria for Early Euthanasia-Rodent
330 Postoperative Care - Rodent
#343 Surgical Prep/Rident/Recovery Surgery

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

CHANGES Staff added and removed; new drug and study added, gavaging added; mice added; animal numbers have increased by 110.

c.c. Approved Protocol - D. Hill, E. Arany, D. Forder
Approval Letter - E. Arany, D. Forder

University Council on Animal Care • The University of Western Ontario
Animal Use Subcommittee • Health Sciences Centre • London, Ontario • N6A 5C1 • Canada

The UNIVERSITY *of* WESTERN ONTARIO – COUNCIL ON ANIMAL CARE

PROJECT RENEWAL APPLICATION

Please complete the following using your computer. Submit via email to aus@uwo.ca
Send signed original Section I. (Authorization) with supporting documents to Rm. 510, MSB

A. PROJECT/GRANT/INVESTIGATOR INFORMATION - *Mandatory Completion Required*

Investigator Name: DR. David J. Hill	**Department:** Medicine, Physiology		**Institution:** Lawson Health Research Institute	
Office Address: **Office Telephone:**	**Lab Address:** H434 **Lab Telephone:** 64309		**Email Address:** dhill@lri.sjhc.london.on.ca	
Project Title: *This is a new title*- Yes ☐ Pancreatic beta cell renewal in development: Implications in Diabetes				
Grant Title *(if different from Project title)*: *This is a new title*- Yes ☐ same			**Current Protocol #:** 2007-116-12	
Previous Project or Grant Title *(if above project/grant title is new)*: same				
Funding Source: Corporate/Contract **Name:** CIHR			**Source Grant #**	**OR** **Applied for:** ☐
Renewal to take effect immediately	YES: ☒	NO: ☐	**If no, please specify date:**	mm / dd / yy / /

B. MAJOR CHANGES AT RENEWAL - *Mandatory Completion Required*

Use the drop-down lists to indicate all overall change types requested within this renewal form		
A change in...	**Major Area of Change**	**Provide Justification & Other Details Not Added to Related Sections**
	Personnel - Sect.E	
	Training Requirements - Sect.E	
	Other	
	Click Here	
	Click Here	

	Click Here	

C. PROGRESS - *Mandatory Completion Required*

DESCRIBE PROGRESS TO DATE *Use as much space as necessary*
Three manuscripst are ready to be submitted. Results were pepresented in numerous international meetings. Three times oral presentations. Post-doctoral fellow was awarded twice travel awards. Abstracts were presented At The American Endocrine Society(2003-2004) Canadian Diabetes Association (2004), International Endocrine Society (ICE) (2004).

D. ANIMAL STATUS – *Mandatory Completion Required*

Animal Status *Please respond to all ...*	*Yes* OR *No*	If yes, please provide An explanation...	If yes, please indicate measures which can or are being taken to resolve this...
1. Did animals in this project die (not euthanized), or were animals euthanized prior to expected survival time?	No		
2. Did animals exhibit signs of more severe pain or stress than outlined in the protocol?	No		
3. Did animals develop conditions or diseases unrelated to your research?	No		
4. If analgesics were indicated therapeutically in the protocol, please answer the following:			
i. Were the analgesics administered?			No
ii. Was the frequency as indicated?			N/A
iii. Were any problems encountered with the administration of the analgesic?			N/A
THE PROVISION OF ADEQUATE ANALGESIA TO RESEARCH ANIMALS IS OF VITAL CONCERN. PLEASE CONTACT ACVS IF YOU HAVE QUESTIONS OR CONCERNS REGARDING CLINICAL SITUATIONS.			

Complete List of All Staff Currently Working under This Protocol

Please note that any staff NOT listed here will be removed from the protocol and have all related privileges revoked.

Complete All						If Yes to Animal Work, complete this section																				
						Species *M=Mouse, R=Rat,* RB=Rabbit, P=Pig, SG=Sheep/Goat, C=Canine, NP=Primate, OR=Other Rodent, O=Other If Other, provide detail below									Procedures 1=Basic Handling 2=Health Monitoring 3=Blood Collection 4=Injections 5=Anaesthesia 6=Surgery-Recovery 7=Surgery-Non-Recovery 8=Euthanasia/Post Mortem 9=Other, provide detail below								
First Name	Last Name	Position *Researcher Staff Student*	If Emergency Contact Person, Enter After Hours Contact Number	Email Address	Hands On Animal Work? Yes Or No	M	R	RB	P	SG	C	NP	OR	O	1	2	3	4	5	6	7	8	9
Catherine M.	Beamish	Staff		mbeamish@uwo.ca	Yes	☒	☒	☐	☐	☐	☐	☐	☐	☐	☒	☒	☒	☒	☒	☐	☐	☒	☐
Astrid	Chamson-reig	Student		achamson@lri.sjhc.london.on.ca	Yes	☒	☒	☐	☐	☐	☐	☐	☐	☐	☒	☒	☒	☐	☐	☐	☐	☐	☐
Kelly	Weese	Student		kweese@uwo.ca	Yes	☐	☒	☐	☐	☐	☐	☐	☐	☐	☒	☒	☒	☐	☐	☐	☐	☒	☐
Brenda	Strutt	Staff		bstrutt@lri.sjhc.london.on.ca	Yes	☒	☒	☐	☐	☐	☐	☐	☐	☐	☒	☒	☒	☒	☒	☐	☐	☒	☐
Edith	Arany	Researcher	438 7934	earany@lri.sjhc.london.on.ca	Yes	☒	☒	☐	☐	☐	☐	☐	☐	☐	☒	☒	☒	☒	☒	☐	☐	☒	☐
Mike	Nicholson	Student			Yes	☐	☒	☐	☐	☐	☐	☐	☐	☐	☒	☐	☒	☐	☐	☐	☐	☐	☐
Aaron	Cox	Student		arcox@uwo.ca	Yes	☒	☒	☐	☐	☐	☐	☐	☐	☐	☒	☒	☒	☒	☒	☐	☐	☒	☐
Sandra	Thyssen	Student		sthyssen@lri.sjhc.london.on.ca	Yes	☐	☒	☐	☐	☐	☐	☐	☐	☐	☒	☒	☒	☒	☒	☐	☐	☒	☐
David J.	Hill	Researcher		dhill@lri.sjhc.london.on.ca	No	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
		Click Here			N/A	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
		Click Here			N/A	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
		Click Here			N/A	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Provide Other Detail Here:		A. Chamson, B.Strutt, E Arany,D. Hill, S.Thyssen will review the animal course in listed in the webpage.				M.Nicholson, Kelly Weese and Aaron Cox are taking the animal course now.																				

"Please submit documentation related to previous animal training with the renewal signed original"

F. EXPERIMENTAL CHANGES - *Mandatory Completion Required*

1. Changes in Experimental Design *Major changes require the submission of a new "Application for the Use of Animals in Research and Teaching"*	
Change Type: *List all applicable changes*	**Provide Justification and Other Change Details Not Added to Related Sections:**
Procedures	Addition of a new Drug (atorvastatin) 20 mg/kg dailyfor 15 days by gavage (1 ml)
Procedures	Gavage done by Kelly Weese, Mike Nicholson and Dr. Edith Arany (properly trained by Vet. services)
Procedures	Treat pregnant rats during last trimester of pregnancy and lactation (15 days in total)
Procedures	Pups of these rats undergo STZ treatment as done previously I.P. at 4 day 4 (one dose of 70 mg/kg)
Procedures	Pups sacrificed at 14 days of age as stated before
Procedures	Glucose monitoring before sacrifice by EdithArany, Kelly Weese, Kathie Beamish and Mike Nicholson(from rat tail vein, 2 ul)

G. BREEDING

The AUS has authorized the integration of breeding with related research protocols.	
I. Is breeding involved within this protocol?	☒ Yes ☐ No If Yes, please complete II. To VII. in ***lay terms***:
II. Provide justification for maintaining a breeding colony	1)We will breed the rat rats in our facility to determine exact time of pregnancy. 2)to provide enough animals that express g the gene of interest
III. Provide justification for breeding numbers	Breeding is needed to maintain the colony
IV. List procedures used in the breeding colony	Ear tatoos and DNA sampling from the ear tissues
V. Number estimation and use of surplus animals (those not required for experimental programs, or retired breeders)	**Surplus Number Estimate:** 500 **Surplus Use:** 50
VI. Breeding colony location, if different from research housing.	South Street campus
VII. Research associates directly involved in the care of animals in this breeding colony	**Name:** Astrid Chamson-Reig **Contact #:** 64309 **Email:** achamson@lri.sjhc.london.on.ca **Name:** D.J.Hill **Contact #:** **Email:** dhill@lri.sjhc.london.on.ca

2. New SOPs Required by Experimental Design Changes Described in E. 1. *Indicate SOPs not previously documented, but involving the above mentioned experimental changes. For more detailed information, go to the following web page:* http://www.uwo.ca/animal/website/VS/Content/SOPs.htm By listing these SOPs you are indicating willingness to comply with their contents in this project.
100 Monitoring/Tumour Growth/Rodents
310 Holding Period Post-Admission
321 Criteria for Early Euthanasia/Rodents
360 Blood Collection/Volumes/Multiple Species
320 Euthanasia
Click Here
Click Here

H. ANIMAL REQUIREMENTS - *Mandatory Completion Required*

Animals Required for Upcoming Year Only: *Mandatory completion required*

Species:	*Strain &/or Other Species Detail*	*Age/ Weight*	*Sex*	*Pain Level*	*Used in Breeding ?*	*Total Animal #s*
Rat	Wistar	200	Female	C	Yes	60
Rat	Wistar	200	Male	C	Yes	10
Mouse	FVB/N(transgenic)	preg/litter	Male/Fema	C	Yes	30
Mouse	FVB/N(transgenic)	adult	Male	C	Yes	15
Mouse	FVB/N(wild type)	preg/litter	Male/Fema	C	Yes	30
Mouse	FVB/N(wild type)	adult	Male	C	Yes	15
Click Here			Click Here	Click Here	Click Here	
Click Here			Click Here	Click Here	Click Here	
Click Here			Click Here	Click Here	Click Here	
Click Here			Click Here	Click Here	Click Here	

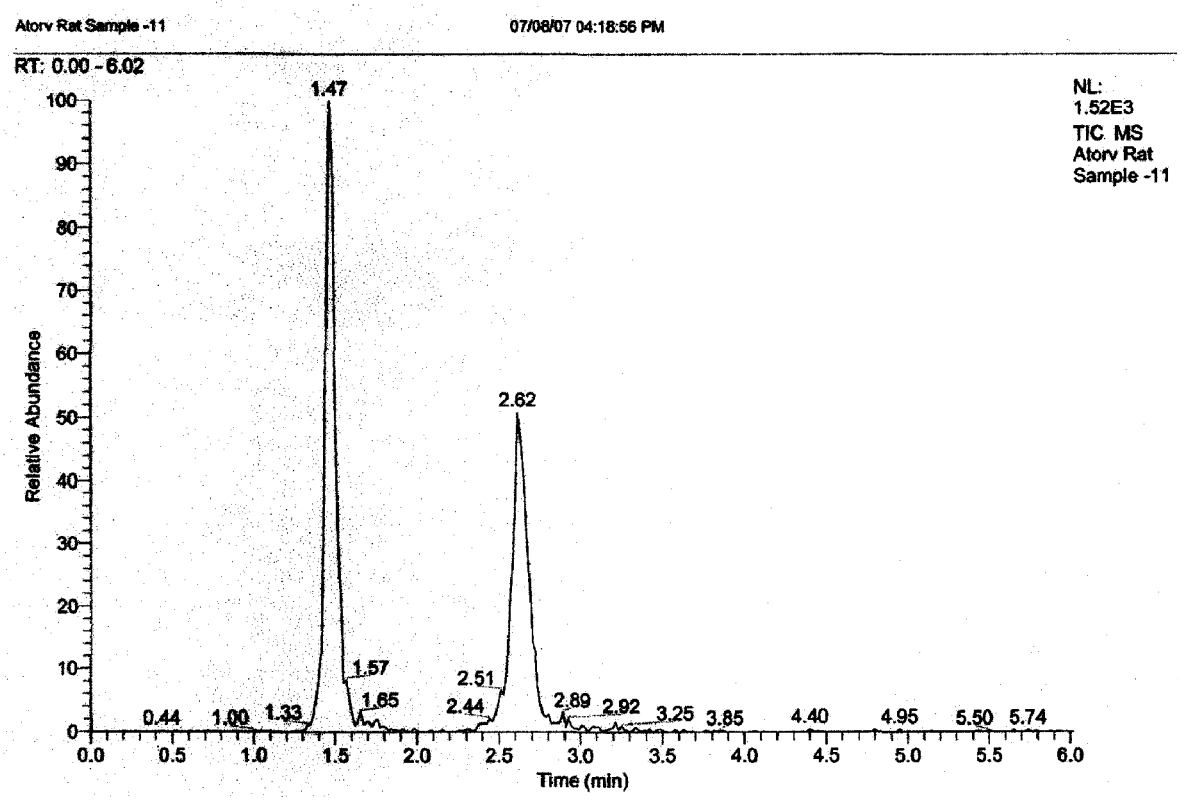
I. DECLARATION *Mandatory Completion Required*

All animals used in this research project will be cared for in accordance with the recommendations of the Canadian Council on Animal Care and the requirements of the provincial legislation entitled, "The Animals for Research Act," of the Province of Ontario.

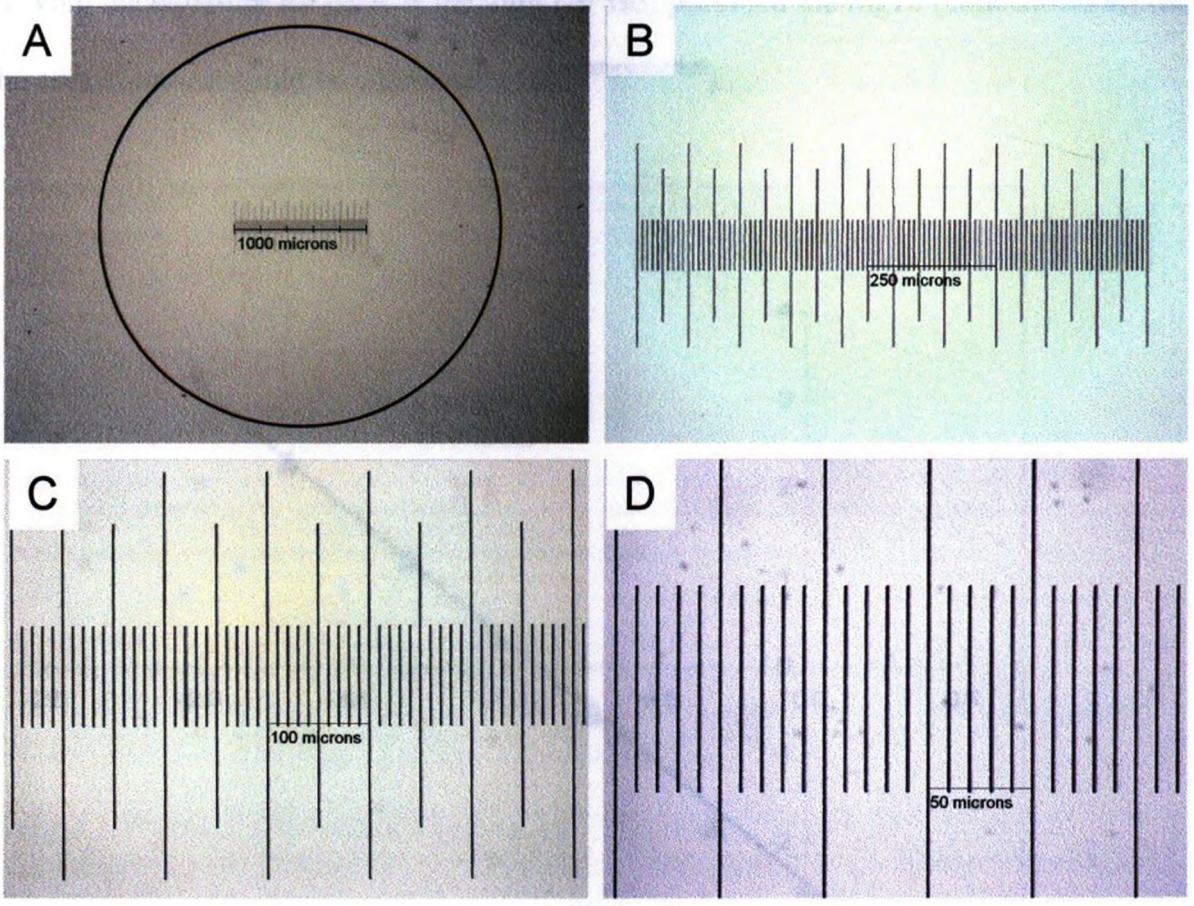
Please Sign & Date Below

Protocol #: *(Same as Section A)* **2002-114-12**	
Principal Investigator or Course Director **Print Name:** **Signature:**	**Date** Dec 7, 2004
AUS Office Use Only **APPROVAL OF ANIMAL USE SUBCOMMITTEE**	

Appendix 2. A chromatogram from the HPLC/MS indicating the relative abundance of the internal standard, RS and sample, AT at their retention times of 1.5 and 2.6 respectively. The retention time was used to identify the standard and sample. The max signal was $1.52*10^3$ ions (100% relative abundance). The MS normalized the strength of the other signals to the max signal and this was measured as relative abundance.



Appendix 3. Photomicrographs of a calibration slide using a Zeiss (A) 2.5X (B) 10X (C) 20X and (D) 40X objective lens in order to calibrate the microscope to the Northern Eclipse Version 7.0 computer software. The correct pixel count that corresponded with the electronic calibration bar was determined at each magnification.



Appendix 4. The standard curve for the insulin RIA was the logit-log plot. The x-axis was log dose and y-axis was logit dose. The logit-log calculation was determined by a transformation of the data in order to linearize the standard curve by using logit B/Bo vs. $\log_{10}$ concentration, where: logit (B/Bo) = Ln ((B/Bo)/ (1-B/Bo)) with B = corrected counts per minute (cpm) (blank subtracted) for each tube, and Bo = maximum binding. Solving the equation for each of the logit (B/ Bo) provided the log10 (concentration) then the concentration could be calculated ($10^{\log \text{ concentration}}$).