

**Imaging Pancreatic β -cell *in vivo* using
Manganese-Enhanced Magnetic Resonance Imaging**

By

Li-Wen Lee

Metabolic and Molecular Imaging Group

MRC Clinical Sciences Centre

Imperial College London

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Abstract

Diabetes is characterized by absolute or relative deficiency of insulin secretion by β -cells. Currently, there are no non-invasive diagnostic tools for assessing β -cell mass and function *in situ*. This thesis aims to develop and implement MRI techniques to image the β -cell *in vivo*. Calcium ion (Ca^{2+}) entry occurs during insulin secretion and the manganese ion (Mn^{2+}) has been used as a Ca^{2+} surrogate to study Ca^{2+} transport in β -cells. Mn^{2+} is also a positive T_1 contrast agent and imaging $[\text{Mn}^{2+}]$ changes with manganese-enhanced MRI (MEMRI) may be used to monitor Ca^{2+} influx during insulin secretion. I hypothesize activated β -cells take up more manganese than resting cells after manganese chloride (MnCl_2) administration; therefore, the glucose-stimulated pancreas may show higher signal intensity (SI) than the non-stimulated pancreas by T_1 -weighted MRI.

Being thin and diffuse, the mouse pancreas is difficult to image. It was found to be best delineated by magnetization prepared rapid gradient echo (MP-RAGE) MRI. However, MP-RAGE is not conventionally used for quantitative studies and the relationship between MP-RAGE data and $[\text{Mn}^{2+}]$ have to be determined. First, an *in vitro* study was performed and showed a positive correlation between the effective R_1 (R_1 -effective) values and $[\text{Mn}^{2+}]$. Then, SI profiles and R_1 -effective values at increasing Mn^{2+} doses were obtained in the pancreas. Additionally, there was a linear correlation between tissue $[\text{Mn}^{2+}]$ by inductively-coupled plasma atomic emission spectrometry and R_1 -effective by MP-RAGE. The results showed that the MP-RAGE sequence can be used in a semi-quantitative manner.

Subsequently, the methodology was applied to image the pancreas *in vivo*, with and without glucose challenge, in healthy and streptozotocin-induced diabetic mouse models. It revealed a statistically greater signal in the glucose-stimulated pancreas compared to control in healthy mice but not in diabetic mice. Further, Mn^{2+} infusion appeared to have minimal effects on blood glucose levels and islet morphology. Muscle glucose uptake is also a Ca^{2+} -regulated process and therefore MEMRI was applied in the muscle. Results showed increased manganese uptake in glucose-stimulated muscle, suggesting MEMRI may be used for monitoring muscle glucose uptake.

This thesis demonstrates an *in vivo* methodology to detect enhanced Mn^{2+} influx in the activated pancreas and skeletal muscle, opening up opportunities for assessing β -cell and skeletal muscle function during normal and abnormal glucose homeostasis.

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Declaration of Contributors

The majority of the work contained in this thesis was performed by the author. Any assistance and collaboration is detailed below:

Optimization of magnetization-prepared rapid gradient echo sequence

Optimization of magnetization-prepared rapid gradient echo sequence was carried out with the assistance of Dr Anthony Price.

Tissue manganese analysis

Analysis of tissue manganese content by inductively coupled plasma atomic emission spectrometry and laser ablation inductively coupled plasma spectrometry was carried out in collaboration with Prof. Cameron McLeod, Sheffield University. Analysis of tissue manganese content by X-ray fluorescence was carried out in collaboration with Dr Po-Wah So.

Islet histology

H&E and immunohistochemistry stains of pancreatic islets were carried out in collaboration with Dr Huw Jones, AstraZeneca Pharmaceuticals.

Abbreviations

$[Ca^{2+}]_i$	intracellular calcium ion concentration
^{11}C	carbon-11
^{18}F	fluorine-18
^{18}F -FDG	fluorine-18 labeled fluorodeoxyglucose fluorodeoxyglucose
3D	three-dimensional
AAS	atomic absorption spectrometry
ADP	adenosine diphosphate
AMPK	adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
Ba^{2+}	barium ion
BAT	brown adipose tissue
Ca^{2+}	calcium ion
Cd^{2+}	cadmium ion
C-peptide	connective peptide
CT	computerized tomography
DMT1	divalent metal transporter-1
DTBZ	dihydrotetrabenazine
Fe^{3+}	iron (III) ion

FOV	field of view
Gd ³⁺	gadolinium ion
GFP	green fluorescent protein
GLUT	glucose transporter
GLUT-2	glucose transporter-2
H&E	hematoxylin and eosin Y
HbA _{1C}	hemoglobin A _{1C}
i.p.	intraperitoneal
i.v.	intravenous
ICP-AES	inductively coupled plasma atomic emission spectrometry
IR	inversion recovery
IR-SE	inversion recovery spin-echo
K _{ATP} channel	ATP-sensitive potassium channel
MEMRI	manganese-enhanced magnetic resonance imaging
Mn	manganese
Mn ²⁺	manganese ion
Mn ³⁺	manganese (III) ion
MnCl ₂	manganese chloride
MnDPDP	mangafodipir trisodium
MnSOD	manganese superoxide dismutase

MP-RAGE	magnetization-prepared rapid gradient echo
NMR	nuclear magnetic resonance
NOD mouse	non-obese diabetic mouse
OPT	optical projection tomography
PET	positron emission tomography
PPAR γ	peroxisome proliferator-activated receptor- γ
PP	pancreatic polypeptide
RF	radiofrequency
ROI	region of interest
SE	spin-echo
SI	signal intensity
SI ₀	baseline signal intensity
SPECT	single photon emission computerized tomography
SPIO	superparamagnetic iron oxide
STZ	streptozotocin
TD	delay time
TE	echo time
TfR	transferrin receptor
TI	inversion time
TI _{null}	inversion time at null point
TR	repetition time

TR ₀	overall repetition time
USPIO	ultrasmall superparamagnetic iron oxide
VGCCs	voltage-gated calcium channels
VMAT2	vesicular monoamine transporter-2
WAT	white adipose tissue
WHO	World Health Organization
XRF	X-ray fluorescence
Zn	zinc
Zn ²⁺	zinc ion

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1 Introduction

1.1 Brief overview

Assessment of the β -cell by non-invasive methods is important for the research and development of novel therapies for diabetes. β -cells are the major cell type in islets of Langerhans. However, islets constitute only 1-2 % of the pancreatic mass and are distributed throughout the pancreas. Further, it is difficult to distinguish β -cells from surrounding non- β -cells within the islets by current imaging modalities. Therefore, β -cell imaging represents a challenge and there is a need to introduce targeted imaging agents to highlight the β -cell for imaging.

Glucose is the main stimulator for β -cell insulin secretion. An increase in blood glucose e.g. following feeding leads to uptake of glucose by β -cells via the glucose transporter-2 (GLUT-2) (Unger, 1991). Increased glucose metabolism elevates the cytoplasmic adenosine triphosphate (ATP) to adenosine diphosphate (ADP) ratio, closing ATP-sensitive potassium (K_{ATP}) channels by the binding of ATP to the β -cell K_{ATP} channel subunits, and causing cellular depolarization. Depolarization induces calcium influx through voltage-gated calcium channels (VGCCs) which leads to exocytosis of insulin granules in the β -cell. There is a positive correlation between glucose-stimulated insulin secretion and an increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$).

The manganese ion (Mn^{2+}) is a T_1 contrast agent in MRI and can act as a surrogate of the calcium ion (Ca^{2+}), and thus can be used to monitor Ca^{2+} fluxes across the cell membrane. Manganese-enhanced MRI (MEMRI) has been used to image Ca^{2+} -related cellular activity in neurons and myocardium. As exocytosis of insulin granules from β -cells is triggered by cellular Ca^{2+} influx, MEMRI may be used to image β -cell function.

1.2 Pancreas

The pancreas is a lobulated glandular organ located between the stomach, duodenum, large intestine, left kidney and spleen. In humans, the pancreas is a solid retroperitoneal organ divided into 3 parts: head, body and tail with no distinct margin between the parts. It is generally considered that the head of the pancreas is located between the duodenum and superior mesenteric vein whereas the rest of the pancreas is equally divided into the body and tail (distal part) (Suda *et al.*, 2006). The gross anatomy of the rodent pancreas differs from that of the human (Figure 1-1). In rodent, the pancreas is a rather thin, membrane-like, diffuse organ (Case, 2006). It is divided into three parts: duodenal, biliary and gastrosplenic portions (Kara, 2005). The biliary portion is the central part of the pancreas located between the biliopancreatic duct and descending duodenum. The duodenal pancreas is the part of the pancreas extending into the duodenal ligament. The gastrosplenic pancreas is the left part of the pancreas extending along the dorsal part of the stomach towards the splenic hilum. The gastrosplenic portion is the biggest and thickest portion of the rodent pancreas.

The pancreas consists of two types of tissues: the endocrine (islets of Langerhans) and exocrine tissues (Figure 1-2). The exocrine pancreas gives rise to the vast majority of the pancreatic mass and comprises primarily of acinar cells which secrete digestive enzymes such as lipases, proteases and nucleases into the pancreatic ductal system. The endocrine pancreas or the islets of Langerhans are small nests of cells (100-500 μm in size) scattered throughout the pancreas (Medarova and Moore, 2009; Quesada *et al.*, 2008). Pancreatic islets makes up only 1-2 % of the total pancreas mass (Figure 1-2) and each islet consists of 1000-3000 cells (Quesada *et al.*, 2008).

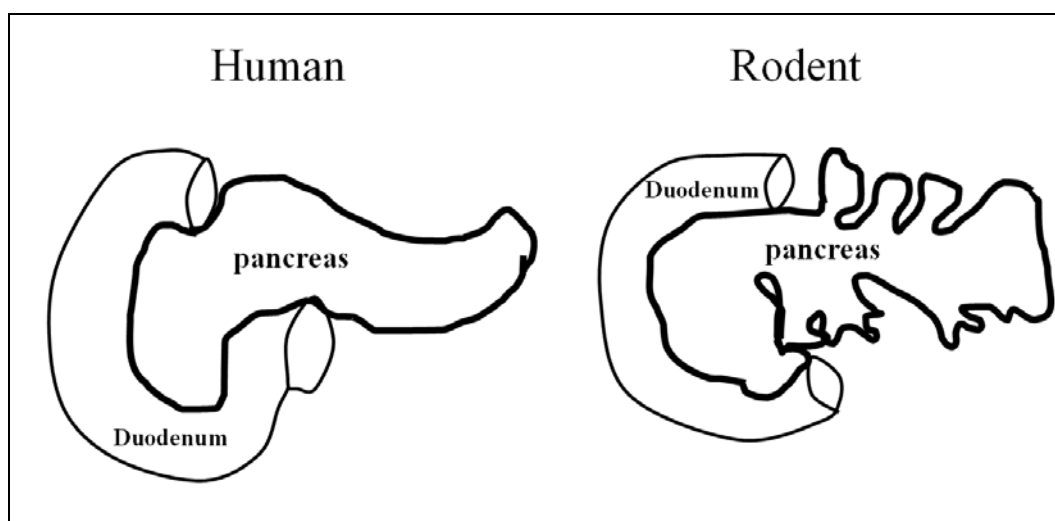


Figure 1-1. Illustration of the human and rodent pancreas.

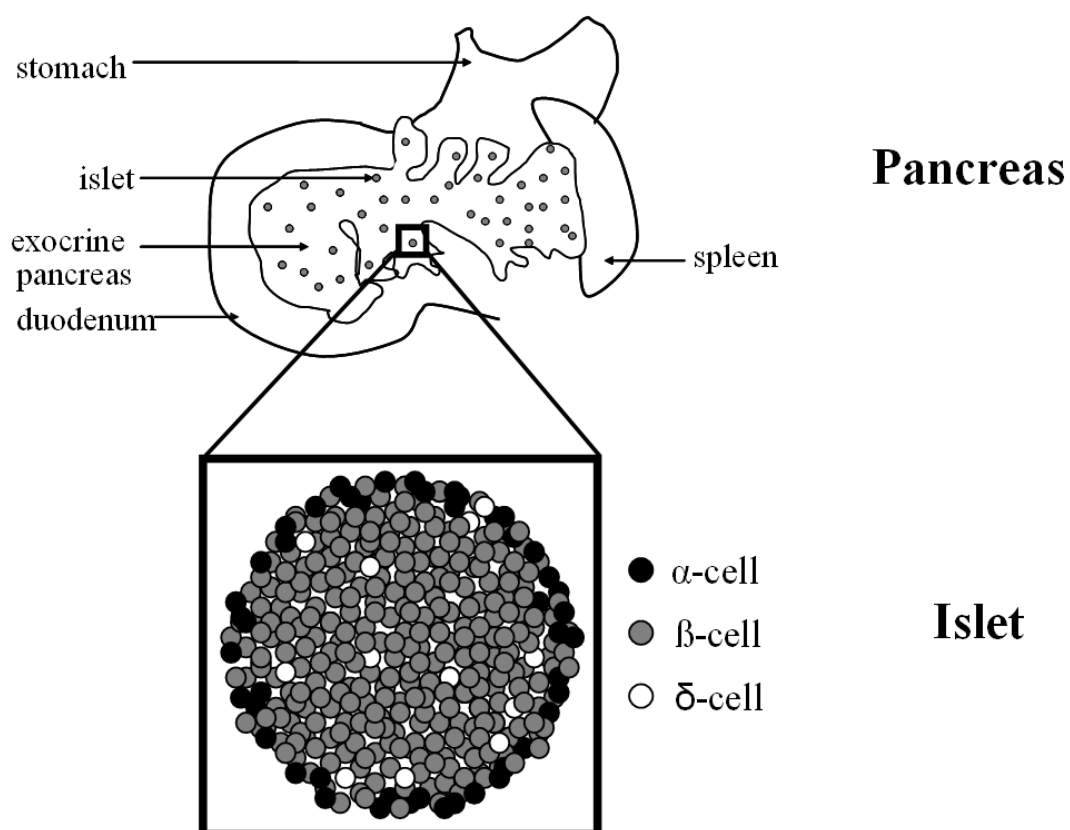


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1.3 The islets of Langerhans

There are four major cell types in the islets of Langerhans: α -cells, β -cell, δ -cells and pancreatic polypeptide (PP) cells secreting glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. The islet architecture in rodent and human are markedly different. Mouse β -cells are located in the core and non- β -cells in the perimeter (Figure 1-2) whereas human α -, β - and δ -cells are distributed throughout the islet (Brissova *et al.*, 2005).

The islets of Langerhans make up only 1-2 % of the total pancreatic mass but obtain 10 % of the total pancreatic blood flow (Jansson and Hellerstrom, 1983). Indeed, the islet perfusion is disproportionately greater than its relative volume in the pancreas. Within a mouse islet, the patterns of islet flow are either from periphery to inner core to perfuse the β -cell first or from one side to another to perfuse islet cells regardless of cell type (Nyman *et al.*, 2008). Thus, the β -cell either has the priority or equal priority to the rest of the other islet cell types. Glucose is the major regulator of β -cell function, although the effect of glucose on β -cell or islet perfusion remains unclear. An increase in the pancreatic blood flow by 25 % was demonstrated while islet blood flow increased by 80 % at 25 min after intraperitoneal (i.p.) glucose injection in rats by using a non-radioactive microsphere technique, (Jansson and Hellerstrom, 1983). However, when glucose was given intravenously, pancreatic blood flow was unaffected by glucose while islet blood flow doubled (Jansson, 1988).

1.3.1 The β -cell

The β -cell is the predominant cell type in the islet, making up 60-80 % of all islets (Edlund, 2002; Mu *et al.*, 2006) and there are around 2000 β -cells in each islet (Meier *et al.*, 2006). β -cells produce insulin following stimulation, e.g. by glucose. The major function of insulin is to lower glucose levels in response to hyperglycaemia i.e. by facilitating glucose uptake into peripheral tissues and inhibiting hepatic glucose production. Regulation of blood glucose levels requires adequate numbers and good quality β -cells to sense the blood glucose concentrations appropriately, and hence the concept of the functional β -cell mass. Loss of pancreatic β -cell mass and function plays an important role in the pathogenesis of diabetes.

1.3.1.1 Insulin

Insulin is the major hormone regulator of glucose metabolism whereas glucose is the primary regulator of insulin synthesis and exocytosis. Insulin was first discovered by Frederick Banting, Charles Best and John James Rickard Macleod in 1921. Since then, injection of insulin has been used for the treatment of diabetes. To acknowledge their breakthrough achievement in the field of diabetes, the Nobel Prize in Physiology or Medicine was awarded to Banting and Macleod in 1923. Insulin is synthesized exclusively by the β -cell and stored in secretory granules in the resting state. Upon stimulation by e.g. hyperglycaemia, the insulin secretory granule is transported to the β -cell surface and insulin released by exocytosis. Insulin synthesis and exocytosis is primarily regulated by glucose, although also regulated by the complex interplay between glucose metabolism and a variety of factors such as nutrients, incretin, neural

innervation and autocrine-paracrine signalling (Berggren and Leibiger, 2006). Insulin secretion is pulsatile with major pulses which occur every 1.5-2 h and the most significant pulses after food intake (Polonsky *et al.*, 1988). A circadian rhythm of insulin secretion has also been reported (Boden *et al.*, 1996; Polonsky *et al.*, 1988).

1.3.1.2 Calcium signalling

Ca^{2+} signalling plays a major role in both normal cellular function and pathological processes in most cell types. Such signalling requires an increase of cytoplasmic Ca^{2+} in response to different stimuli and is coupled to a wide variety of cellular responses including exocytosis, contraction, metabolism, transcription, fertilization and proliferation (Berridge *et al.*, 2003). It is well-known that the redistribution of Ca^{2+} stores from cellular organelles and cellular influx of extracellular Ca^{2+} contribute to the elevation in $[\text{Ca}^{2+}]_i$. Ca^{2+} -storing organelles include the endoplasmic reticulum, mitochondria, Golgi apparatus, endosomes, lysosomes and secretory vesicles (Rizzuto and Pozzan, 2006). Since the internal stores have a limited capacity, cellular entry of Ca^{2+} drives most Ca^{2+} -dependent cellular events (Parekh, 2006).

Entry of Ca^{2+} can be either excitation-dependent or excitation-independent. Neurons, muscle cells, and neuroendocrine cells are electrically excitable cells (Dolphin, 2006). Ca^{2+} enters across the plasma membrane via VGCCs which open when these cells are depolarized. The remaining cell types such as epithelial, endothelial and blood cells, are electrically non-excitable and Ca^{2+} entry pathways are activated by inositol triphosphate receptors (Berridge, 2005; Putney, 2001). Calcium-sensitive fluorescent indicators can be used to measure $[\text{Ca}^{2+}]_i$ and monitor Ca^{2+} -signalling pathways in

whole cells, optically. Intracellular electrodes or patch-clamp techniques can also be used to record cellular electrical activity (Petersen *et al.*, 2005).

It is difficult to distinguish whether an increase in cytoplasmic Ca^{2+} derives from release of internal stores or from Ca^{2+} influx across the plasma membrane. One method for observation of Ca^{2+} influx is to employ Ca^{2+} surrogates such as the Mn^{2+} or barium ion (Ba^{2+}) and then to monitor their intracellular accumulation during cellular activation. Mn^{2+} is the most commonly used ion for studying Ca^{2+} influxes since Mn^{2+} can pass through many subtypes of Ca^{2+} channels in the plasma membrane (Hsiao and Payne, 1998). Further, it is not normally present in cells and can bind Fura-2, a fluorescent Ca^{2+} indicator, to quench fluorescence. Therefore, Ca^{2+} entry can be estimated by measuring Mn^{2+} -induced quenching of Fura-2 fluorescence in the presence of extracellular Mn^{2+} . Owing to the ability of Mn^{2+} to act as a surrogate for Ca^{2+} , combined with the paramagnetic property of Mn^{2+} , MEMRI can be used as a novel route to map Ca^{2+} -related cellular activities.

The pancreatic β -cell is an electrically excitable cell in which Ca^{2+} signalling plays a critical role in insulin secretion (Rorsman, 1997). Hyperglycaemia induces increased β -cell uptake of glucose and subsequent glucose metabolism leads to a rise in the cytoplasmic ATP/ADP ratio. This causes closure of K_{ATP} channels in the plasma membrane and so the β -cell undergoes depolarization, which leads to opening of VGCCs and the influx of extracellular Ca^{2+} . The increase in $[\text{Ca}^{2+}]_i$ triggers exocytosis of the insulin-containing granules. The stimulation of β -cells with glucose induces a biphasic insulin secretion *in vivo*. It is of great interest that the mouse islets *in vitro*

display a similar first-phase insulin secretion but not the second-phase (Nunemaker *et al.*, 2006). Therefore, there is a need for non-invasive observation of pancreatic β -cell *in vivo* to assess β -cell mass and function.

1.3.2 The α -cell

The α -cell is the second most abundant cell type in the pancreatic islet and constitutes 15-20 % of islet cells (Gopel *et al.*, 2000). It secretes glucagon in response to hypoglycaemia and is a counter-regulatory hormone of insulin. Glucagon induces liver glycogenolysis and gluconeogenesis which results in an increase in the blood glucose level (Quesada *et al.*, 2008). Pancreatic α -cells are electrically-excitabile cells equipped with Ca^{2+} -channels and Ca^{2+} entry induces glucagon secretion by α -cells (Gromada *et al.*, 1997; Quesada *et al.*, 2008).

1.3.3 The δ -cell

The δ -cell secretes somatostatin in response to glucose stimulation and the mechanism of glucose sensing is similar to that in β -cells (Kanno *et al.*, 2002), although, little is known about this process. It is suggested that the release of somatostatin by δ -cells is also Ca^{2+} -dependent and associated with an increase in $[\text{Ca}^{2+}]_i$ (Bermudez-Silva *et al.*, 2009; Braun *et al.*, 2009).

1.4 Diabetes

1.4.1 Aetiology

Diabetes is a group of metabolic disorders characterized by high blood glucose levels. It is estimated that expenditure on diabetes is around £9 billion/year or 10 % of the NHS budget in the UK (Diabetes UK, 2009) and \$US 116 billion in the US (American Diabetes Association, 2007). The actual costs due to diabetes exceed direct health expenditure as there are indirect costs such as income loss and reduced life quality. Therefore, it is vital to explore both the prevention and cure for diabetes. There are two common types of diabetes: type 1 and type 2 diabetes.

1.4.1.1 Type 1 diabetes

Type 1 diabetes accounts for 10-15 % of patients with diabetes. It is caused by β -cell destruction, leading to a reduction in β -cell mass and absolute deficiency of insulin secretion. When 80 % of the insulin production capacity is lost, hyperglycaemia develops. Thus, diabetes can only be diagnosed when β -cell mass loss exceeds a critical threshold i.e. when 90 % of the β -cell mass has been destroyed (Martinic and von Herrath, 2008). By such time, anti-diabetic interventions will only alleviate symptoms and not cure diabetes.

β -cell destruction is caused by an autoimmune response associated with a recruitment of antibodies to islet cell cytoplasm, insulin, glutamic acid decarboxylase, tyrosine phosphatases IA-2 and IA-2 β in the vast majority of type 1 diabetes patients (Atkinson

and Eisenbarth, 2001). This immune-mediated form of diabetes is classified as type 1A diabetes according to the report of American Diabetes Association (American Diabetes Association, 2003). This form of diabetes is associated with multiple genetic and environmental factors and the individual is also prone to other autoimmune diseases. Type 1B diabetes (idiopathic type 1 diabetes) is characterized by β -cell destruction without the presence of autoimmune antibodies and is inheritable (Pinero-Pilona and Raskin, 2001), although its aetiology remains unclear.

1.4.1.2 Type 2 diabetes

Type 2 diabetes affects 85-90 % of people with diabetes and is the most common type of diabetes. In health, the pancreatic β -cell secretes insulin in response to elevated blood glucose to maintain glucose homeostasis by facilitating glucose uptake in skeletal muscle and adipose tissue, and decreasing hepatic glucose production. Whenever normal amounts of insulin produce a less than normal biologic response, insulin resistance occurs (Kahn, 1978). Insulin resistance can arise from either genetic factors or can be acquired. With the aggravation of insulin resistance, β -cells increase insulin secretion to adapt to the increased insulin demands. Failure to compensate for insulin resistance is fundamental to the pathogenesis of type 2 diabetes. Therefore, insulin levels in type 2 diabetes patients can be elevated, normal, or high; depending on the stage of the disease. Thus, the resulting hyperglycaemia in type 2 diabetes is a consequence of relative rather than absolute insulin deficiency.

Type 2 diabetes is a heterogeneous disorder characterized by disorders of insulin secretion and action. Many factors such as age, obesity and physical inactivity contribute to it. Progression from insulin resistance to the onset of diabetes may take 10-20 years (Codario, 2005; Hawkins and Rossetti, 2005) or may never happen (Lebovitz, 1999). Insulin resistance appears to progress to type 2 diabetes only if β -cell function is inadequate to compensate for the insulin resistance.

1.4.2 Current diagnostic methods

The diagnosis of diabetes is based on the clinical symptoms and laboratory tests. Current laboratory tests include measurements of blood glucose, insulin, connecting peptide (C-peptide) and hemoglobin A_{1C} (HbA_{1C}).

1.4.2.1 Blood glucose measurement

1.4.2.1.1 In man

The most common method for diagnosing diabetes is the fasting plasma glucose test. It reflects the ability of β -cells to lower blood glucose. The plasma glucose test reflects β -cell activity to some extent and is an indirect marker of β -cell function, although a relatively late marker of diabetes. Fasting plasma glucose levels are measured after overnight (usually 8 h) fast.

Another way of assessing β -cell function is the glucose tolerance test which measures the glucose clearance rate from the blood after a glucose load. In humans, it is routinely

performed after oral ingestion of 75 g of glucose and blood glucose is measured 2 h after dosing. The 2006 World Health Organization (WHO) recommendation report outlined a clear definition for both abnormal blood levels and glucose tolerance test (Table 1-1) (World Health Organization 2006).

Diabetes	
Fasting plasma glucose	≥ 7.0 mM (126 mg/dL)
or 2 h plasma glucose*	≥ 11.1 mM (200 mg/dL)
Impaired glucose tolerance (IGT)	
Fasting plasma glucose	< 7.0 mM (126 mg/dL)
and 2 h plasma glucose*	≥ 7.8 and < 11.1 mM (140 and 200 mg/dL)
Impaired fasting glucose (IFG)	
Fasting plasma glucose	6.1 – 6.9 mM (110 - 125 mg/dL)
and (if measured)	
2 h plasma glucose*	< 7.8 mM (140 mg/dL)

Table 1-1. WHO recommendations for human diabetes. *Venous plasma glucose 2 h after ingestion of 75g oral glucose load.

1.4.2.1.2 In mice

Unlike in humans, there are no such clear description of glucose levels for both the fasting plasma glucose and glucose tolerance tests in diabetic mouse models. A review article shows diabetes was indicated by non-fasting blood glucose levels > 200 mg/dL

in 3 %, > 250 mg/dL in 18 %, > 300 mg/dL in 27 %, > 350 mg/dL in 37 %, > 400 mg/dL in 37 % and > 450 mg/dL in 32 % of reports (Melzi *et al.*, 2007).

In mice, there are two common forms of glucose tolerance tests: the oral glucose tolerance test and the intraperitoneal (i.p.) glucose tolerance test. In mice, the glucose tolerance test is routinely performed following an overnight fast to achieve baseline blood glucose level. Then, mice are challenged with an i.p or intravenous (i.v.) glucose load: blood glucose levels are measured prior to glucose administration and at 15, 30, 60 and 120 min after glucose administration. An abnormal glucose tolerance test is normally diagnosed by comparison with a control group. Glucose doses from 0.5 to 3 g/kg body weight and the most common dose being 2 g/kg body weight have been used for the glucose tolerance test (Andrikopoulos *et al.*, 2008).

1.4.2.2 Blood insulin measurement

The insulin precursor is a single-chain protein containing the B chain and A chain of insulin linked through a C-peptide segment (Steiner, 1969). The insulin precursor is then converted into insulin, a two-chain peptide consisting of A and B chains, by the loss of C-peptide (Steiner *et al.*, 1967). Measurement of peripheral insulin levels serve as a gold standard for assessing β -cell secretory activity (Cavaghan and Polonsky, 2005). However, 50 % of insulin is removed during first-pass metabolism by the liver (Polonsky *et al.*, 1983) making insulin measurement an ineffective indicator for β -cell function.

1.4.2.3 Blood levels of connecting peptide

C-peptide is a by-product of insulin production (see above), secreted by β -cells in equimolar quantities with insulin. Unlike insulin, C-peptide is not removed by the liver during first-pass metabolism and thus, is a more reliable marker of functional β -cell mass and a useful measurement to distinguish type 1 from type 2 diabetes (Marques *et al.*, 2004; Vinik, 2004).

1.4.2.4 Blood levels of hemoglobin A_{1C}

HbA_{1C} is formed by the irreversible binding of glucose molecules to the N-terminal of β -chains of haemoglobin A (Bunn *et al.*, 1976). It is slowly formed during the 120-day life span of an erythrocyte such that it reflects an average glucose level over the preceding 6-8 weeks (Cooppan, 2005). The HbA_{1C} test provides an indicator for long-term glucose control and complications in diabetic patients rather than as a measurement of β -cell function.

1.4.3 Treatment

In clinical practice, patients with type 1 diabetes (absolute insulin deficiency) are treated with exogenous insulin, pancreas or islet transplantation. The treatment strategies for type 2 diabetes are far more complex, including diet control, exercise, administration of blood glucose lowering drugs, and/or insulin. Lowering of blood glucose can be achieved by increasing insulin secretion, lowering insulin resistance or enhancing glucose disposal and production. For example, sulfonylureas bind

sulfonylurea receptor subunits of β -cell K_{ATP} channels, causing closure of K_{ATP} channels, cellular depolarization and Ca^{2+} entry with the increase in intracellular Ca^{2+} stimulating insulin secretion (Edwards and Weston, 1993). On the other hand, metformin exerts its anti-diabetic effect through increasing glucose disposal and decreasing hepatic glucose output (Bailey and Turner, 1996). The mechanism of thiazolidinediones to treat diabetes is incompletely understood but appears to work through binding to nuclear receptors, peroxisome proliferator-activated receptor- γ (PPAR γ) (Pittas and Greenberg, 2002). PPAR γ is involved in glucose and lipid metabolism and its activation leads to increased uptake of fatty acids and lipogenesis in adipose tissue which in turn lower levels of circulating fatty acids and lower insulin resistance (Ovalle and Fernando, 2002).

1.4.4 Experimental models of diabetes

1.4.4.1 *In vivo* models

1.4.4.1.1 Type 1 diabetes

Type 1 diabetes is characterized by the destruction of insulin-secreting β -cells secondary to autoimmune reaction or to other unknown aetiologies. Animal models of type 1 diabetes can be generated by destruction of β -cells by chemicals, e.g. streptozotocin (STZ) or induction of autoimmune responses.

Streptozotocin (STZ)-induced diabetic mice

STZ is a β -cell-specific mycotoxin produced by *Streptomyces achromogenes* var. *streptozoticus* and possesses antineoplastic, antibiotic, diabetogenic and mutagenic activities (Reusser, 1971). STZ has been a well-established method of inducing β -cell necrosis resulting in a deficiency of insulin secretion and generating an appropriate model to study type 1 diabetes (Wang and Gleichmann, 1998). However, the mechanism why β -cells are selectively sensitive to STZ is not fully understood (Konrad *et al.*, 2001).

The pancreas of STZ-induced diabetic mouse shows peri-islet lymphocyte and macrophage infiltration, permeating islets of Langerhans with islet distortion and β -cell necrosis by histological examination (Like and Rossini, 1976). The same study also shows smaller size and degranulation of the surviving β -cells but normal appearing α - and δ -cells. Among the commonly used mouse strains, non-obese diabetic mice (NOD mice) and C57BL/6 mice are both susceptible to STZ-induced diabetes (Gonzalez *et al.*, 2003). STZ can be given as a single high dose (150-200 mg/kg) or as multiple low doses (40-50 mg/kg in 5 consecutive days) via i.v. or i.p. routes. A single STZ injection causes β -cell necrosis within 4 h after dosing and achieves hyperglycaemia rapidly, whereas multiple dosing induces gradual elevation of blood glucose with a maximal reached during 10-25 days after the last injection (Like and Rossini, 1976).

Non-obese diabetic (NOD) mice

Non-obese diabetic (NOD) mice are genetically susceptible to autoimmune disease (Aoki *et al.*, 2005) and is a useful model to study the mechanisms of β -cell destruction. They can spontaneously develop insulinitis and diabetes but not all progress from insulinitis to diabetes. Insulinitis in NOD mice is characterized by mononuclear infiltration predominantly of CD4⁺ T lymphocytes, but CD8⁺ T lymphocytes, B lymphocytes, and macrophage/dendritic cells are also found (Mathew and Leiter, 2005). The incidence of spontaneous diabetes in NOD mice is 60 % to 80 % in females and only 20 % to 30 % in males (Anderson and Bluestone, 2005).

1.4.4.1.2 Type 2 diabetes

Type 2 diabetes is a heterogeneous and multifactorial disease associated with both insulin resistance and impaired insulin secretion. Several attempts have been made to develop mouse models of type 2 diabetes, including by dietary and genetic manipulation.

Diet-induced type 2 diabetes models

A high fat diet has been shown to induce diabetes and obesity in various rodent strains (Collins *et al.*, 2004). The C57BL/6 mouse is commonly used in type 2 diabetes studies, because it is genetically disposed to develop obesity, hyperglycaemia, hyperinsulinemia and insulin resistance, parallelling the progression of human type 2 diabetes (Seldin *et al.*, 1994). Insulin resistance and visceral fat accumulation develop after being fed a high fat diet containing 40-60 % of calories for 8-16 weeks (Reuter,

2007). Further, the increase in body fat deposition is consistent with the concomitant development of insulin resistance and hyperglycaemia (Black *et al.*, 1998).

Genetically manipulated models

Although type 2 diabetes is a polygenic disorder, monogenic mouse models of diabetes are still valuable as they can provide information about the mechanisms in the pathophysiology of diabetes. Insulin resistance in type 2 diabetes can be achieved by manipulating a number of genes, including those encoding the insulin receptor, GLUT-4, IRS1-4 and Akt present in a number of tissues (LeRoith and Gavrilova, 2006). Mouse models of obesity, such as the *db/db* and *ob/ob* mice, are associated with type 2 diabetes. The *db/db* mouse has a point mutation in the leptin receptor gene, displaying hyperglycemia and hyperlipidemia (Matveyenko and Butler, 2006). The *ob/ob* mouse, on the other hand, has a point mutation in the leptin gene and also displays hyperglycaemia and hyperlipidemia (Matveyenko and Butler, 2006). In response to hyperglycaemia, there is an increase in islet size and number in the *ob/ob* mouse resulting in enhanced insulin secretion compensating for the insulin resistance (Bock *et al.*, 2003). In the *db/db* mouse, there is a transient increase in insulin secretion followed by a decrease in β -cell mass due to islet necrosis (Shafrir *et al.*, 1999).

The limitation of using rodent type 2 diabetes models is that they do not show similar islet pathology to that in humans, such as production of islet amyloid (Cefalu, 2006). Another important difference is the capacity of rodent islets to proliferate compared to human islets (Parnaud *et al.*, 2008). Although rodent models fail to reproduce all

aspects of human type 2 diabetes, they are still of great value, in mimicking certain pathophysiological aspects of human type 2 diabetes.

1.4.4.2 *In vitro* models

1.4.4.2.1 Isolated islets

Isolation of islets from the rodent pancreas was first described by Lacy and Kostianovsky and most subsequent islet isolation procedures designed have been based on this pioneering work (Lacy and Kostianovsky, 1967). Briefly, the animal is anesthetized and the pancreas perfused with Hanks buffer through the common bile duct. Then, the pancreas is removed, cut into small pieces and digested with collagenase. Islets are isolated from this tissue preparation by sedimentation or centrifugation. During the isolation process, islets are exposed to a variety of cellular stresses, e.g. disruption of cell-matrix relationship and loss of peri-islet basement membrane, leading to islet damage (Rosenberg *et al.*, 1999; Wang *et al.*, 1999). Prolonged islet culture has been shown to reduce responsiveness to glucose stimulation and maintenance of islets in culture has remained a challenge (Gaber *et al.*, 2001).

1.4.4.2.2 Cultured β -cell lines

Common β -cell lines, such as RIN lines, HIT lines, β TC lines, MIN lines, β H9C lines and NIT-1 line, are derived mainly from insulinomas and preneoplastic islet cells in rodents (Ulrich *et al.*, 2002). Therefore, results derived from β -cell lines may need to be confirmed in primary cultured islets since differences may exist between normal and

transformed cells. Most of the β -cell lines secrete insulin in response to glucose stimulation (Hohmeier and Newgard, 2004) and are appropriate models for studying the mechanism of glucose-stimulated insulin secretion. However, transformed β -cell lines can cause a decreased insulin response after prolonged culture (Ulrich *et al.*, 2002).

1.5 Insulin target tissues

Blood glucose levels remain in a narrow range between 4 and 7 mM under physiological conditions and this tight regulation is determined by a balance between glucose production by the liver and glucose disposal by peripheral tissues which are mediated by insulin production by β -cells (Ruderman *et al.*, 2005). Target tissues for insulin action include the liver, skeletal muscle and adipose tissue. Glucose is membrane-impermeable and its transport across cell membrane requires the glucose transporter (GLUT) family. There are five GLUT proteins, GLUT-1, 2, 3, 4, and 5, which differ in their properties and tissue distributions. GLUT-2 occurs primarily in the liver, pancreatic β -cell, intestine and kidney, whereas GLUT-4 is located primarily in heart, skeletal muscle and adipose tissue (Shepherd and Kahn, 1999). Increased blood glucose levels leads to increased glucose uptake in β -cells through GLUT-2. The rise in intracellular glucose stimulates insulin-gene transcription in the β -cell as well as increased glycolysis leading to insulin secretion (Efrat *et al.*, 1991).

1.5.1 Skeletal muscle

Skeletal muscle is the major glucose-utilizing organ responsible for 75 % of insulin-dependent glucose uptake following an exogenous glucose load (Saltiel and Kahn, 2001). Skeletal muscle glucose disposal *in vivo* is regulated by glucose delivery to muscle cells, GLUTs and intracellular glucose metabolism (Rose and Richter, 2005). Glucose supply to muscle cells is dependent on skeletal muscle perfusion and blood glucose levels. Muscle blood flow increases during exercise and is known to increase in a linear manner in response to muscle workload (Andersen and Saltin, 1985).

Therefore, muscle contracture is a potent stimulator of muscle glucose uptake. During exercise, muscle contracture causes depletion of muscle glycogen stores and increased ATP demand, leading to the activation of adenosine monophosphate-activated protein kinase (AMPK) (Bergeron *et al.*, 1999). Glucose uptake by skeletal muscle can be totally insulin-independent via the activation of AMPK following exercise (Holloszy, 2005).

Glucose uptake into the skeletal muscle cell is mediated by GLUT-4 in the cell membrane. GLUT-4 is stored in intracellular vesicles which translocates to the plasma membrane in response to elevated insulin level. The role of insulin in skeletal muscle is to increase skeletal muscle perfusion (Baron and Clark, 1997) as well as initiate GLUT-4 translocation. Similar to the other exocytotic processes in neurons and pancreatic β -cells, exocytosis of GLUT-4 is mediated by increased $[Ca^{2+}]_i$ (Schudt *et al.*, 1976; Westfall and Sayeed, 1990).

In summary, glucose uptake by skeletal muscle is regulated by insulin-mediated and contraction-mediated glucose transport pathways. Both pathways involve an increase in cytosolic Ca^{2+} with an influx of extracellular Ca^{2+} due to insulin-mediated glucose uptake, and from intracellular Ca^{2+} stores for the contraction-mediated pathway (Lanner *et al.*, 2008). As insulin can induce a net Ca^{2+} influx and Mn^{2+} can quench fura-2 fluorescence (a fluorescent Ca^{2+} indicator) and has been used to detect Ca^{2+} influx in response to insulin activation in skeletal muscle (Lanner *et al.*, 2006), MEMRI may be used to image insulin-mediated glucose transport processes *in vivo* in skeletal muscle.

1.5.2 Liver

The liver is a major site of glucose production which is inhibited by insulin and stimulated by glucagon. Both insulin- and glucagon-mediated processes induce Ca^{2+} influx into hepatocytes *in vitro* (Applegate *et al.*, 1997; Barritt *et al.*, 2008; Benzeroual *et al.*, 2000). Glucagon binds to the glucagon receptor on the cell membrane of hepatocytes and an increase in intracellular Ca^{2+} is accompanied by this process (Hems and Whitton, 1980; Nathan and Cagliero, 2001; Xu and Xie, 2009). However, the role of Ca^{2+} in the suppression of hepatic glucose production by insulin is unclear. Although some studies suggest that insulin and glucagon both facilitate Ca^{2+} influx into hepatocytes (Applegate *et al.*, 1997; Barritt *et al.*, 2008; Benzeroual *et al.*, 2000). Thus, Ca^{2+} -regulated signalling pathways are complex, with numerous Ca^{2+} channels involved in a wide range of cellular functions (Barritt *et al.*, 2008). Unlike in the pancreas (see 1.2) and skeletal muscle (see 1.5.1), the relationship between glucose uptake and hepatic blood perfusion is unclear (Bor *et al.*, 1980; Pugliese *et al.*, 1988).

Mn^{2+} is metabolized in the liver. In addition, Mn^{2+} also plays an important role in the enzyme function in the liver. Mn^{2+} is known to be a constituent of mitochondrial MnSOD, liver arginase and liver pyruvate carboxylase; and an activator of glutamine synthetase and alkaline phosphatase (Christianson and Cox, 1999; Greger, 1998; Hurley *et al.*, 1959; Thunus and Lejeune, 1999).

The liver plays a major role in the regulation of blood glucose levels by balancing uptake and production of glucose (Nordlie *et al.*, 1999). Blood glucose is mainly derived from digested nutrients in the fed state and is maintained by hepatic glucose

production in the fasted state (Kruger *et al.*, 2006). In the liver, glucose is produced via the pathways of gluconeogenesis and glycogenolysis. The rate of gluconeogenesis remains constant while the rate of glycogenolysis changes in different metabolic states in healthy human (Nuttall *et al.*, 2008). In type 1 diabetes, the absolute insulin deficiency results in the lack of a suppressive effect on hepatic glucose production and a deficiency of peripheral glucose uptake (Kruger *et al.*, 2006). However, relatively little is known about the role hepatic glucose production in patients with diabetes (Nuttall *et al.*, 2008).

1.5.3 Adipose tissue

Adipose tissue is a metabolic and endocrine organ. It expresses receptors for insulin, glucagon, growth hormone, thyroid-stimulating hormone, glucagon-like peptide-1 and secretes proteins such as leptin, adiponectin, adipisin, tumor necrotic factor α , interleukin-6 (Kershaw and Flier, 2004). There are two types of adipose tissues in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT). The white adipocyte contains a single large lipid droplet and a peripherally located nucleus whereas the brown adipocyte contains multilocular lipid droplets and a centrally located round nucleus (Hull, 1966). WAT is found subcutaneously, around visceral organs and in the bone marrow. BAT is lobulated, located in deep cervical regions including the interscapular, suprasclavicular and paravertebral regions. WAT is the primary site of energy storage and plays a role in metabolic regulation and physiological homeostasis (Trayhurn and Beattie, 2001). BAT is important in the regulation of energy expenditure (Cypess *et al.*, 2009).

WAT and BAT also appear to have different origins with WAT being derived from stromal vascular fraction and BAT being obvious to have a similar lineage to skeletal muscle (Enerback, 2009). BAT in adults human was not thought to be present but recent Positron Emission Tomography (PET)-Computerized Tomography (CT) scan conducted in both lean and obese adult has provided compelling evidence of BAT (Cypess *et al.*, 2009).

In WAT, the role of Ca^{2+} influx in insulin-mediated glucose uptake remains unclear. Some studies suggest that intracellular Ca^{2+} is essential for insulin-mediated glucose transport and GLUT-4 translocation in isolated white adipocytes (Draznin *et al.*, 1988; Pershadsingh *et al.*, 1987; Worrall and Olefsky, 2002). Although other studies suggest that increased $[\text{Ca}^{2+}]_i$ is not related to insulin-mediated glucose transport in isolated white adipocytes *in vitro* (Kelly *et al.*, 1989; Khil *et al.*, 1997).

In isolated rat epididymal adipocytes, Mn^{2+} has been shown to be insulinomimetic by enhancing insulin binding (Baquer *et al.*, 2003; Ueda *et al.*, 1984) and Mn^{2+} deficiency can result in impaired glucose transport and defects in lipid metabolism (Baly *et al.*, 1990; Davis *et al.*, 1990). It has been shown that Mn^{2+} treatment was able to reverse declining insulin sensitivity in diabetic rat model (Baquer *et al.*, 2003). Therefore, it is possible that there is a link between exposure to Mn^{2+} and insulin resistance in the adipose tissue.

In BAT, the relationship between insulin and GLUT-4 mediated glucose seems similar to that in WAT (Cannon and Nedergaard, 2004) and the role of Ca^{2+} influx in insulin-mediated glucose uptake is still not fully established.

1.6 β -cell imaging

Autopsy studies show there is a 90 % β -cell mass loss in type 1 diabetes and 0-65 % in type 2 diabetes (Matveyenko and Butler, 2008). It is essential to accurately image the β -cell mass before irreversible loss of β -cells leads to the symptoms of diabetes to provide an earlier therapeutic window and so, increase the likelihood of maintaining the β -cell mass. In spite of the rapid progress in biotechnology, there is no accurate and non-invasive diagnostic tool for assessing β -cell mass and function *it situ*. Currently, direct and reliable measurements for β -cells are limited to biopsy or autopsy studies. Therefore, there is a need to develop imaging techniques to image the β -cell anatomy, pathophysiology and metabolism *in situ* to improve understanding of diabetes. Current imaging technologies used for β -cell imaging include optical imaging, nuclear imaging and MRI (Saudek *et al.*, 2008). These modalities all require the use of β -cell-specific imaging probes for the quantification of β -cell mass.

Although the dominant cell type in the islet, the β -cell only comprises 1 % of the total pancreatic volume. Therefore, potential imaging technologies must have the ability to effectively identify β -cell signal intensity (SI) from that of the background pancreas. Two characteristics of an ideal β -cell imaging probe has been established: (1) a 1000:1 β -cell labelling specificity with respect to exocrine cells (2) signal from bound probes in β -cells is significantly higher than that from unbound probes in extracellular spaces (Sweet *et al.*, 2004).

1.6.1 Optical Imaging

Optical imaging is an imaging method which uses visible or near infrared radiation. It has limited penetration and so is unable to visualize the pancreas unless the pancreas is surgically exposed. Thus, optical imaging methods cannot be readily translated to the clinic as compared to nuclear imaging and MRI. However, confocal microscopy, a high-resolution optical imaging technique, has been used to generate three-dimensional (3D) images from a selected tissue focal plane *in vivo*.

Confocal fluorescent microscopy is capable of imaging fluorescently labelled cells or molecules (Balas, 2009). Operating in the ultraviolet range, it has the advantage of cellular to subcellular resolution. On the other hand, it has the drawbacks of low penetration depth (20-200 μm) and high background signal. Recently, a novel application of *in vivo* imaging of islet morphology, vascularization, cell function and cell survival *in vivo* was reported using confocal laser scanning microscopy (Speier *et al.*, 2008). The investigators transplanted green fluorescent protein (GFP)-expressing islets into the anterior chamber of the eye which was used as a natural body window to overcome the depth limitation of confocal microscopy. This technique allows non-invasive imaging of GFP-expressing islets over time. By administration of Texas red, a fluorescence dye via the tail vein, the islet vasculature could also be readily visualized.

Optical projection tomography (OPT) is an approach for imaging of small specimens with 1-10 mm thickness (Holmberg and Ahlgren, 2008). It acquires nearly cellular level resolution ($\sim 15\text{-}20\ \mu\text{m}$), generating 3D morphology of a particular organ or

distribution of labelled cells (Holmberg and Ahlgren, 2008; Sharpe, 2004). Alanentalo *et al.* reported an OPT-based *ex vivo* 3D quantitative method to demonstrate the complex shape of mouse pancreas and visualize insulin-labelled islet distribution and volume in NOD mice (Alanentalo *et al.*, 2007). This work also demonstrated a correlation between the total islet volume and onset of type 1 diabetes in a NOD mouse model. Transplanted islets has been detected *in vivo* by both MRI and fluorescence microscopy by labeling islets with superparamagnetic iron oxide (SPIO) nanoparticles and near-infrared fluorescent Cy5.5 dye (Medarova *et al.*, 2005).

1.6.2 Nuclear imaging

Radionuclides undergo radioactive decay or disintegration with the emission of ionizing radiation in the form of α , β or γ rays. Often compounds are radiolabelled and allow visualization of pathophysiological processes (Imam, 2005). A gamma camera is used to detect waves or particles emitted by radiolabelled complexes in tissues to generate images after administration into the subject. The detected radioactive signals are then processed mathematically to create images to show the location as well as activity of the radiolabelled metabolite or radiopharmaceutical. Current nuclear imaging modalities include Single Photon Emission Computerized Tomography (SPECT) and PET. The disadvantages of nuclear imaging are poor spatial resolution and the use of ionizing radiation.

1.6.2.1 SPECT

The most common radionuclides used in SPECT are technetium-99m, thallium-201, gallium-67, iodine-123, iodine-131 and samarium-153 which emit gamma rays during radioactive decay. SPECT cameras are designed to detect the single photons emitted by gamma-emitting radionuclides (Powsner and Powsner, 2006b). SPECT is less expensive than PET, although relatively less sensitive and of lower spatial resolution. By labelling β -cells with a radiolabeled IC2 antibody (a proposed β -cell-specific antibody), Moore *et al* showed a good correlation between β -cell mass and accumulation of radioligands *in vivo* using SPECT (Moore *et al.*, 2001).

1.6.2.2 PET

Common radionuclides used in PET are carbon-11(^{11}C), nitrogen-13, oxygen-15, fluorine-18 (^{18}F). These radionuclides undergo positron decay, producing paired photons traveling away in opposite directions (Powsner and Powsner, 2006a). PET scanners are designed to detect these paired annihilation photons. Biological molecules are radiolabelled by replacement of nuclei with photon emitting nuclei e.g. ^{18}F labeled fluorodeoxyglucose (^{18}F -FDG). Specific receptors can be imaged by labeling of appropriate ligands, such as ^{11}C labeled dihydrotetrabenazine (DTBZ) for the vesicular monoamine transporter-2 (VMAT2). Currently probes proposed to image β -cells are ligands targeted to VMAT2, antibodies to zinc transporter-8, and antibodies to β -cell surface proteins.

Monoamines, such as epinephrine, norepinephrine, dopamine, serotonin and histamine, are stored in secretory granules of neurons, endocrine and neuroendocrine cells. The role of VMATs is to transport and package monoamines from the cytoplasm into storage organelles. VMAT2 is located in islets, neurons and chromaffin cells of the adrenal medulla (Freeby *et al.*, 2008). In the pancreas, VMAT2 is found to be expressed specifically in β -cells and not in exocrine cells by immunohistochemistry and *in situ* hybridization (Anlauf *et al.*, 2003). Therefore, it has been proposed as a molecular target for β -cell imaging and measuring the uptake of radioactive ligands that selectively binds VMAT2 represents the insulin secretory capacity (Harris *et al.*, 2008). DTBZ is a specific ligand which binds to VMAT2. Studies showed an excellent correlation between ^{11}C -DTBZ uptake and the β -cell mass in animal models (Simpson *et al.*, 2006; Souza *et al.*, 2006). DTBZ can also be labeled with ^{18}F to give ^{18}F -labeled 9-fluoropropyl-(+)-DTBZ which has been used for mapping the β -cell mass in rats (Kung *et al.*, 2008). It is worth noting that a recent study showed that VMAT2 is expressed in both pancreatic β -cells and PP cells in human (Saisho *et al.*, 2008) such that ^{11}C -DTBZ uptake may not be wholly specific to β -cells (Malaisse *et al.*, 2009).

Zinc ion (Zn^{2+}) is involved in a wide variety of physiological processes in many tissues. It is stored with insulin in the secretory granule and therefore important metal in the β -cell (Hutton *et al.*, 1983). The distribution of Zn^{2+} is high in the β -cell (Clifford and MacDonald, 2000), but also in the brain (Que *et al.*, 2008). Thus, radioactive Zn^{2+} may be used to label the insulin granules in the β -cell to assess insulin storage and secretion. However, insulin granules can be secreted from the β -cell and so lower accumulation of

radioactive zinc (Zn) may be more correlated to a decrease in the insulin store rather than a reduction in the β -cell mass (Schneider, 2008).

1.6.3 MRI

1.6.3.1 Introduction

MRI is a popular medical imaging tool in clinical practice providing excellent soft tissue contrast by appropriate choice of the MRI sequence and parameter values. MRI is more attractive than other non-invasive imaging modalities, as it can be easily translated to the clinic and does not employ ionizing radiation, although MRI is usually more expensive and requires longer scan times. For preclinical research, small animal MRI affords spatial localization of morphological and functional changes in tissues which has become increasingly important (Pautler, 2004b).

MRI exploits the phenomenon of nuclear magnetic resonance (NMR). NMR results from the introduction of charged nuclei into a magnetic field and their adoption of specific orientations. The simplest nucleus is the proton and water protons are the most abundant nuclei in biological tissues (McRobbie *et al.*, 2007). Protons are randomly oriented in the absence of a magnetic field and adopt one of two orientations in the presence of magnetic field, either parallel with the magnetic field (spin up) or antiparallel (spin down). As there is a slight excess of the spin up population, there is a net magnetization (M_0) in the same direction as the applied magnetic field (the z-direction). Application of a radiofrequency pulse at the appropriate frequency will tip the magnetization away from the z-axis (Figure 1-3). The magnetization, M , has both

an M_z and an M_{xy} component. As M returns back to the equilibrium position, M_0 , the rate at which the M_z and M_{xy} components returns to the equilibrium position is termed longitudinal and transverse relaxation, respectively. (Hashemi *et al.*, 2003b). The rate of longitudinal and transverse relaxation is given by the values of T_1 and T_2 , respectively. By T_1 -weighted MRI, tissues with short T_1 can be preferentially positively enhanced. Tissues with short T_2 values can be preferentially negatively enhanced by T_2 -weighted MRI.

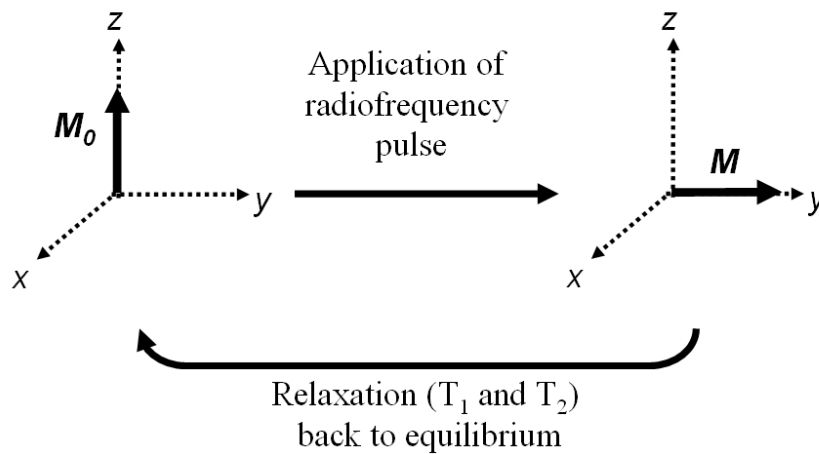


Figure 1-3. The effect of radiofrequency pulse on the bulk magnetization M_0 .

It is challenging to image rodent pancreas by *in vivo* MRI. Firstly, the pancreas is surrounded by organs of the gastrointestinal tract such as the stomach, duodenum, jejunum and colon such that physiological movements can give rise to image blurring. Secondly, the rodent pancreas is a diffuse organ and the voxel in each acquired slice may have a mixture of tissues which contribute to partial volume averaging. Therefore, slice thickness needs to be as thin as possible to avoid partial volume artefacts. Thirdly,

the pancreas is enclosed by solid organs including the liver, left kidney and spleen such that sufficient tissue contrast is needed between the pancreas and its surrounding organs to readily identify their boundaries. Taken together, the criteria for optimal pulse sequences for rodent pancreatic MRI are: short scan times to attenuate the motion artefacts, high spatial resolution to avoid partial volume averaging and high tissue contrast to delineate the pancreas.

It is even more of a challenge to specifically image the β -cell mass and function. Firstly, the volume of the β -cell mass is only 1 % of the pancreas and is scattered throughout the whole pancreas. Secondly, the size of β -cells is smaller than the commonly used spatial resolution of MRI technology. Moreover, the signal from β -cells cannot be separated from other types of cells in the pancreas by MRI and so, β -cell-specific contrast agents are required to visualize only β -cells by MRI.

1.6.3.2 Contrast agents

MRI contrast agents are metal complexes such as Mn^{2+} , iron (III) ion (Fe^{3+}) and gadolinium ion (Gd^{3+}) which alter T_1 and T_2 relaxation times, leading to an increase in the longitudinal and transverse relaxation rates ($1/T_1$ and $1/T_2$) of the water protons, respectively, in tissue. The efficiency to enhance relaxation rates by the contrast agent is referred as the relaxivity (Eric *et al.*, 2008). The relaxivity of MRI contrast agents depends on the number of unpaired electrons. Mn^{2+} (electron configuration $3d^5$), Fe^{3+} (electron configuration $3d^5$) and Gd^{3+} (electron configuration $4f^7$) are all high spin number ions with 5, 5, and 7 unpaired electrons, respectively. They are paramagnetic in

which the unpaired electrons are aligned parallel with the applied magnetic field. The metal ions in MRI contrast agents can be toxic and its toxicity can be reduced by covalent binding, chelation, polymer conjugation, formation of inclusion complexes and liposome entrapment (Thunus and Lejeune, 1999).

In MRI, the delineation of the area of interest in tissue depends on tissue contrast and imaging resolution (Kuo and Herlihy, 2006). MRI images are affected by a combination of spin density, longitudinal relaxation time T_1 and transverse relaxation time T_2 and other effects (De Graaf, 2007). Therefore, any difference in these properties can be used to enhance tissue contrast in MRI. MRI contrast agents can enhance the tissue contrast between diseased and normal tissues, indicate organ perfusion and evaluate blood flow-related changes in MR images by influencing the relaxation rates of water protons (Caravan *et al.*, 1999). By serial assessment of contrast kinetics following administration of a contrast agent, dynamic contrast-enhanced MRI can provide insights into the integrity and perfusion of the tissue (Choyke *et al.*, 2003), and has been clinically used for differential diagnosis of lesions with different hemodynamic characteristics.

MRI contrast agents can be used as intravascular agents for imaging vascular structures such as Gd-based contrast agents, intracellular agents to image cellular activity such as Mn-based contrast agents, or labelling of biological molecules such as iron oxide-based contrast agents (Thunus and Lejeune, 1999).

1.6.3.2.1 Gd^{3+}

Gd^{3+} is a toxic heavy metal and therefore chelated forms of Gd^{3+} are used for clinical application to reduce toxicity (Caravan *et al.*, 1999; Westmeyer and Jasanoff, 2007). Gd^{3+} -based contrast agents are by far the most commonly used MRI contrast agents with only rare and mild adverse reactions (Caravan *et al.*, 1999). However, recent reports show there is a strong association between exposure to Gd^{3+} -based contrast agents and development of nephrogenic systemic fibrosis (Agarwal *et al.*, 2009; Grobner and Prischl, 2007) and has the disadvantage of being non-biocompatible (Mohs and Lu, 2007). Gd^{3+} -chelates are extracellular contrast agents as they distribute in the plasma and extracellular space. By pre-labelling isolated islets with gadolinium-[10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid] (GdHPDO3A), transplanted islets can be detected *in vivo* up to 65 days post-transplantation in mice (Biancone *et al.*, 2007). However, GdHPDO3A is not a β -cell-specific contrast agent and this method cannot be applied to image native islets *in vivo*.

1.6.3.2.2 Iron oxide nanoparticles

Iron oxide-based contrast agents are iron oxide crystals coated with carbohydrates such as dextran or carboxydextran. Those that are > 50 nm in size are referred to as SPIO particles whereas those that are < 50 nm in size are ultrasmall superparamagnetic iron oxide (USPIO) particles (Erpelding *et al.*, 2008). Iron oxide nanoparticles have a T_2 shortening effect and decrease SI in areas of accumulation on T_2 - or T_2^* -weighted MRI. This class of MRI contrast agent is biocompatible (Bulte and Kraitchman, 2004) and

taken up by Kupffer cells in the normal reticuloendothelial system, primarily in the liver and thus are liver-specific MR contrast agents (Tanimoto and Kuribayashi, 2006). Transplanted islets have been pre-labelled with iron oxide nanoparticles prior to transplantation and have been successfully imaged *in vivo* by MRI (Evgenov *et al.*, 2006). The NOD mouse is a murine model of spontaneous type 1 diabetes characterized by lymphocytic infiltration in β -cells and β -cell destruction (Yang *et al.*, 1997). By labelling lymphocytes with SPIO, the labelled lymphocytes were found in the islet of diabetic NOD mouse *ex vivo* (Moore *et al.*, 2002).

By cell labelling techniques, iron oxide based contrast agents can be used to image transplanted islets or insulinitis. However, iron oxide-labelled islets may be metabolised, released and phagocytosed, e.g. by macrophages. Islets grafted into the kidney capsule and macrophage in the kidney capsule can ingest magnetic nanoparticles (Hsiao *et al.*, 2008), contributing to the SI of islet graft. Therefore, this protocol is not applicable to image native islets, as it is not possible to selectively label native islets *in vivo* in such a way and the clinical application of this technology is limited.

1.6.3.2.3 Mn^{2+}

Manganese (Mn) is an essential cofactor and activating agent to many metalloenzymes in mammalian cells (Christianson, 1997). Mn^{2+} is a constituent of arginase and pyruvate carboxylase, whereas Mn^{2+} and Manganese (III) ion (Mn^{3+}), are key components of manganese superoxide dismutase (MnSOD) (Keen *et al.*, 2000). Therefore, both Mn^{2+} and Mn^{3+} are crucial in biological systems. In addition, free Mn^{2+}

is a MRI contrast agent, predominantly by shortening T_1 relaxation time, giving rise to a positive signal by T_1 -weighted MRI.

Mn may exist in plasma as Mn^{2+} and Mn^{3+} after exposure to $MnCl_2$, as Mn^{2+} can be oxidized to Mn^{3+} (Armstrong, 2008; Aschner *et al.*, 2007). Mn^{2+} and Mn^{3+} are both paramagnetic ions (Schaeffle and Sharp, 2005). Within plasma, Mn^{2+} remains as the free ion or binds to α_2 -macroglobulin (Harris and Chen, 1994), whereas Mn^{3+} binds to transferrin (Rabin *et al.*, 1993). It is known that Mn^{2+} is rapidly taken up by cells and quickly removed from blood after administration (Zheng *et al.*, 2000). An *in vitro* study shows Mn concentrations are high in the liver, pancreas, kidney and adrenal gland, and low in the brain and spleen by inductively coupled plasma atomic emission spectrometry (ICP-AES) after i.v. injection of $MnCl_2$ or MnDPDP (Ni *et al.*, 1997).

Despite its importance, little is known about Mn^{2+} transportation and accumulation in live organisms (Armstrong, 2008; Pittman, 2005). In the cells, Mn^{2+} is accumulated in the mitochondria and nuclei (Kalia *et al.*, 2009). Mn^{2+} can be transported across the plasma membrane of a wide variety of cell types, such as hepatocytes, platelets, endothelial cells, neutrophils, and parotid acinar cells (Merritt *et al.*, 1989; Mertz *et al.*, 1990). Mn has been demonstrated to transport via specific transporters, Ca^{2+} channels and endocytosis (Roth, 2006).

Divalent metal transporter-1 (DMT1) is a proton-dependent transporter for a variety of divalent metal ions including Mn^{2+} , Fe^{2+} , Zn^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Ni^{2+} and Pb^{2+} (Gunshin *et al.*, 1997) but not Ca^{2+} (Xu *et al.*, 2004), and appears to be the major

mammalian Mn^{2+} transporter (Chua and Morgan 1997). Zrt-like, Irt-like protein has been shown to transport Zn^{2+} , cadmium ion (Cd^{2+}) and Mn^{2+} *in vitro* (He *et al.*, 2006). In size, Mn^{2+} has an ionic radius similar to Ca^{2+} ($\text{Mn}^{2+} = 97$ pm, $\text{Ca}^{2+} = 114$ pm) (Shannon, 1976) and therefore, shares some transport pathways, such as VGCCs. Ionic radius is not always important for permeation across VGCCs, so is hydration index, such as with K^{+} channels. In terms of physical and chemical properties, Mn^{3+} is similar to Fe^{3+} and thus TfR which transports Fe^{3+} may also transport Mn^{3+} (Aschner and Gannon, 1994; Davidsson *et al.*, 1989).

Mn^{2+} is metabolized in the liver (Thunus and Lejeune, 1999) and its uptake by the liver has been reported to be a unidirectional process *in vitro* (Keen *et al.*, 2000). The mechanisms of cellular Mn^{2+} influx remain unclear, as well as that of Mn^{2+} efflux (Madejczyk *et al.*, 2009). After entering the liver, Mn^{2+} can either be excreted in the bile (Ni *et al.*, 1997) or accumulate in the mitochondria (Gunter *et al.*, 1975; Gunter *et al.*, 1978).

1.6.3.3 Manganese-enhanced MRI (MEMRI)

The use of Mn^{2+} as a MRI contrast agent can be traced back to the earliest use of MRI (Lauterbur, 1973). Mn^{2+} can act as both an extracellular and intracellular MRI contrast agent as it can exist in the circulation and be transported across cell membranes. There is always a concern when using Mn^{2+} as a contrast agent since Mn^{2+} exposure may lead to respiratory distress, liver failure, cardiac toxicity and neurological disorders (Silva *et al.*, 2004). Mangafodipir trisodium (MnDPDP) is a FDA-approved Mn^{2+} -chelate derived from vitamin B6 to decrease toxicity of Mn^{2+} (Gallez *et al.*, 1996; Regge *et al.*,

2009). MnDPDP releases Mn^{2+} slowly from the chelate and several hours are required for clearance from the blood (Krombach *et al.*, 2004). In contrast, manganese chloride (MnCl_2) is toxic, but has the advantage of fast kinetics and is currently being used in pre-clinical experiments. MnDPDP has been used as a hepatobiliary contrast agent in clinical practice based on its greater affinity to hepatocytes (Elizondo *et al.*, 1991; Lim *et al.*, 1991).

1.6.3.3.1 Brain

Three major applications of MEMRI in the brain are to: detect cellular activity, trace neuronal tracts in visual or olfactory pathways and enhance tissue contrast after a systemic dose (Lee and Koretsky, 2004; Silva and Bock, 2008). Firstly, Mn^{2+} enters into cells through cell membrane VGCCs upon cell depolarisation (Koretsky and Silva, 2004). When cells are activated, Ca^{2+} channels open followed by the Mn^{2+} influx. Thus, cells show greater Mn^{2+} uptake upon activation and this can be observed by T_1 -weighted MRI (Silva *et al.*, 2004). MEMRI has been used to image neuronal activation in the hypothalamus (Kuo *et al.*, 2007; Parkinson *et al.*, 2009), photoreceptors (Berkowitz *et al.*, 2009) and hippocampus (Hsu *et al.*, 2007). Secondly, Mn^{2+} can move along neuronal pathways in an anterograde and retrograde direction after injection into specific brain regions and has been used to trace neuronal networks in animal models (Pautler, 2004a; Silva *et al.*, 2008). Thirdly, increases in tissue contrast are detected on T_1 -weighted MRI following systemic MnCl_2 infusion, aiding better delineation of brain structure (Lin and Koretsky, 1997).

1.6.3.3.2 Heart

Both cellular Ca^{2+} influx and the release of intracellular Ca^{2+} store contribute to the activation of contraction in the heart (Bers and Perez-Reyes, 1999). MEMRI has been demonstrated to act as a potential tool for imaging cellular viability in the myocardium (Brurok *et al.*, 1999) based on the close relationship between $[\text{Ca}^{2+}]_i$ and cardiac contractility (Wendland, 2004). In viable myocardium, the SI increases during the MnCl_2 infusion period, reaching peak enhancement by the end of infusion and remains so for a further 30 min in mice (Hu *et al.*, 2001). Whereas in the infarcted myocardium, Mn^{2+} accumulates during MnCl_2 infusion, but washes out as quick as 3-5 min after the end of infusion (Hu *et al.*, 2004). Moreover, the SI of the myocardium decreases in mice with Ca^{2+} channel blocker and increased with positive inotropic activation following MnCl_2 infusion (Hu *et al.*, 2001), indicating a relationship between Mn^{2+} uptake and cardiac inotropy.

1.6.3.3.3 Pancreas

Pancreatic β -cells are similar to neuronal cells in being electrically excitable cells having VGCCs (Catterall *et al.*, 2005; Ko *et al.*, 1997) and the exocytosis of insulin granules is Ca^{2+} -dependent (Rorsman *et al.*, 2000). Mn^{2+} has been used as a Ca^{2+} surrogate to study Ca^{2+} flux in islets *in vitro* (Rorsman *et al.*, 1982; Rorsman and Hellman, 1983).

During the course of this work, there were two articles in the field of MEMRI of β -cell mass published. An *in vitro* study shows that the SI in Mn^{2+} -labelled islets increase by

200 % following glucose stimulation compared to that of non-stimulated islets by MRI (Gimi *et al.*, 2006). A subsequent *in vivo* study showed that the normalized pancreatic SI is greater in the glucose-stimulated mice compared to control by MEMRI (Antkowiak *et al.*, 2009).

1.6.3.3.4 Lymphocyte

Mn can be taken up by a wide variety of cell types and therefore may be used to label cells. Lymphocytes can be labelled by MnCl_2 for up to 24 h, showing a dose-dependent relationship between changes in T_1/T_2 values and MnCl_2 concentrations *in vitro* (Aoki *et al.*, 2006). However, it is not clear whether the migration of Mn-labelling lymphocytes can be detected *in vivo*.

1.6.3.4 Pulse sequences

A pulse sequence is a sequence of radiofrequency pulses applied throughout the MRI experiment (Hashemi *et al.*, 2003a). Mn^{2+} is a T_1 contrast agent and therefore, T_1 -weighted MRI sequences are employed for performing MEMRI.

1.6.3.4.1 Signal intensity (SI)

The strength of the SI from a given tissue in MRI depends on intrinsic physical parameters of tissue, such as the proton density, T_1 and T_2 , as well as the local magnetic field, field inhomogeneity and choice of imaging parameters (Suranyi *et al.*, 2007). MRI contrast agents are often used to enhance the SI difference between different

tissues, or between normal and diseased tissues. After administration of a T_1 contrast agent, the T_1 value in the tissue where the contrast agent accumulates decreases, leading to an increase in tissue SI by T_1 -weighted MRI. The degree of increased SI depends on the local concentration of the contrast agent. Thus, the pharmacokinetics of the contrast agent in a tissue can be indirectly estimated by analysing the time course of SI changes in a serial set of MR images acquired before and after the administration of a contrast agent. For MEMRI, the signal enhancement can be measured by spin echo (SE) or gradient echo sequences. Only SE and 3D magnetization prepared rapid gradient echo (MP-RAGE) sequences (a gradient echo based sequence), are discussed here.

Spin echo sequence

The SE sequence is the most frequently used MRI pulse sequence. It is made up of a repeated series of 90° pulses to flip the spins into the x-y plane followed by one 180° pulse to eliminate the dephasing effects and generate an echo (Hashemi *et al.*, 2003a). The echo time (TE) is the period between the 90° pulses and the echo whereas repetition time (TR) is the period between two 90° excitation pulses. To perform T_1 -weighted MRI, a value of TE less than 40 ms and a short TR value of less than 750 ms are used whereas for T_2 -weighted MRI, long TE and long TR values are used (Westbrook *et al.*, 2005).

Magnetization-prepared rapid gradient echo (MP-RAGE) sequence

MP-RAGE is a fast scanning technique which acquires high resolution, high contrast, T_1 -weighted images and is used in imaging structural details in the inner ear, skull base

and abdomen in clinical practice (McRobbie, 2007; Mugler and Brookeman, 1991). It acquires 3D image sets of the abdomen with minimal respiratory artefacts (Mugler and Brookeman, 1990a) and so we have proposed its use for mouse pancreatic imaging. The MP-RAGE sequence consists of three steps which constitute the inner loop of the sequence (Figure 1-4): a 180° pre-pulse for magnetization preparation, a repeated small flip angle (α) rapid gradient echo acquisition for data sampling, and a delay for magnetization recovery (Mugler and Brookeman, 1991). A whole MP-RAGE image is constructed by repeating the inner loop of the sequence until all k -space has been collected (Mugler and Brookeman, 1990b).

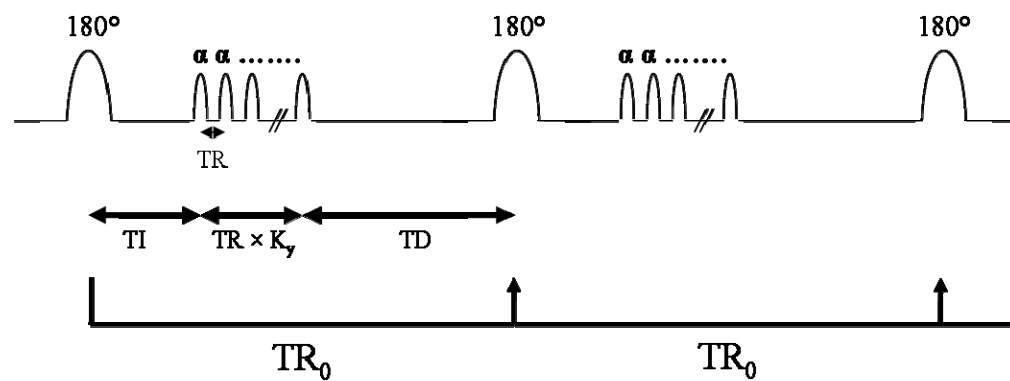


Figure 1-4. Illustration of the MP-RAGE pulse sequence. The inner loop of the MP-RAGE sequence consists of: a 180° inversion pulse followed; a period of inversion time (TI) for magnetization preparation; repetitive small flip angle (α) for data acquisition; and a delay time (TD) for recovery of magnetization. The sequence is repeated to build up the whole imaging data matrix in the k -space. The period between repeated small flip angle pulses is the repetition time (TR). The overall repetition time (TR_0) includes the TI, data acquisition and TD. K_y indicates the number of phase encoding steps in the inner loop.

In MRI, the centre of k -space determines the vast majority of signal in the image and encodes the bulk of image shape, whereas the periphery of k -space contains spatial information which encodes the delineation of fine structures but only weak image signal. Therefore, the time from the centre of inversion pre-pulse to the acquisition of k -space central line in the data acquisition segment controls the SI of different tissues in the MP-RAGE image (Laffon *et al.*, 2001). There are several methods for filling k -space including centric, linear, radial, spiral and parallel ordering (Paschal and Morris, 2004). Centric and linear orderings are two of the most common k -space filling methods used in MP-RAGE sequence (Figure 1-5). With centric ordering, the image acquisition starts from the centre of k -space and then toward the top and bottom of k -space. With linear ordering, the image acquisition starts from top to bottom of k -space. Since some signal recovery occurs during data sampling, the ordering to acquire k -space data can affect accuracy of SI and sharpness of the MP-RAGE image. With linear ordering, the periphery of k -space is acquired first and results in a better delineation of details of the image. Thus, image quality is superior with linear than with centric phase encoding (Mugler and Brookeman, 1991). On the other hand, with centric ordering, the centre of k -space is acquired first and acquired SI is less likely to be affected by the repeated small flip angles.

The image contrast (or SI difference) between two adjacent tissues allows us to delineate the tissue boundary. In MP-RAGE, the inversion preparation pulse is applied to enhance image contrast. Then, a series of small flip angle rapid gradient echo pulses are applied for data collection. MP-RAGE sequence is less susceptible to motion artefacts as it uses very short TR such that the data sampling period ($TR \times Ky$) is relatively short. It needs to be noted that the repeated gradient echo pulses can result in

some signal recovery occurring during data sampling period such that small flip angles are chosen to minimize the effect (Holsinger and Riederer, 1990). Thus, flip angle also plays an important role in image contrast for the tissues of interest (Mugler and Brookeman, 1990). The overall repetition time (TR_0) is used to allow recovery of longitudinal magnetization and prevent saturation effects in MP-RAGE sequence (Jivan *et al.*, 1997).

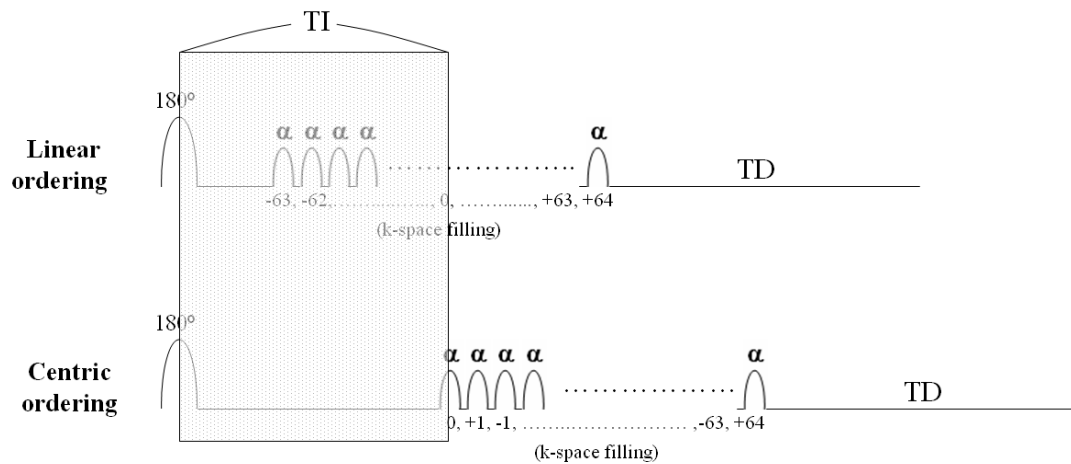


Figure 1-5. Illustration of MP-RAGE sequence with linear and centric k-space filling. The dotted box indicates the inversion time (TI). The grey bars indicate the delay time (TD). The numbers indicate the lines of k-space.

1.6.3.4.2 T_1 measurement

Changes in SI provide a better temporal resolution of Mn^{2+} accumulation compared to changes in T_1 values as the former takes less scan time. However, the MRI SI obtained from the tissue is influenced by experimental conditions, such as imaging parameters

and local magnetic field (Suranyi *et al.*, 2007). Therefore, measurement of T_1 provides a more robust method for determining tissue character. T_1 measurement can be performed by using saturation recovery and inversion recovery (IR) methods. In addition, the MP-RAGE method may also be employed to provide a T_1 value and is discussed (see below).

Inversion recovery method

The IR sequence consists of a 180° pulse to flip the bulk magnetization antiparallel to the static magnetic field, followed by a 90° pulse after a time TI. In IR sequences, recovery of longitudinal magnetization occurs within the time TR, before the next excitation pulse. The time for recovery of 95 % of the longitudinal magnetization is approximately $3 \times T_1$ with about full recovery (99 %) for $5 \times T_1$. The IR method is the gold standard for T_1 measurement. The IR sequence is performed over a range of TI values and fitting the SI at different TIs to equation below (Gowland and Stevenson, 2003):

$$SI(TI) = A - B \times e^{-TI/T_1} \quad \text{----- Equation 1}$$

When TR is $\gg 5T_1$, the equation 1 can be simplified to equation 2.

$$SI(TI) = A \times (1 - 2e^{-TI/T_1}) \quad \text{----- Equation 2}$$

SI (TI) is signal intensity collected at TI

A and B are constants

MRI images are magnitude images and so SI is always positive (magnitude data). However, polarity-restored data are needed for fitting the signal recovery curve to estimate the T_1 value. Therefore, the polarity of the modulus data acquired by inversion recovery sequences needs to be restored before fitting to the signal recovery curve (Figure 1-6). The point at which SI is equal to zero is defined as the null point and the inversion time at the point is denoted as TI_{null} . TI_{null} is $\sim 0.693 \times T_1$. When MRI contrast agents are used, there is a positive correlation between the degree of contrast concentration and the SI obtained by T_1 -weighted sequences at $TI \geq TI_{null}$.

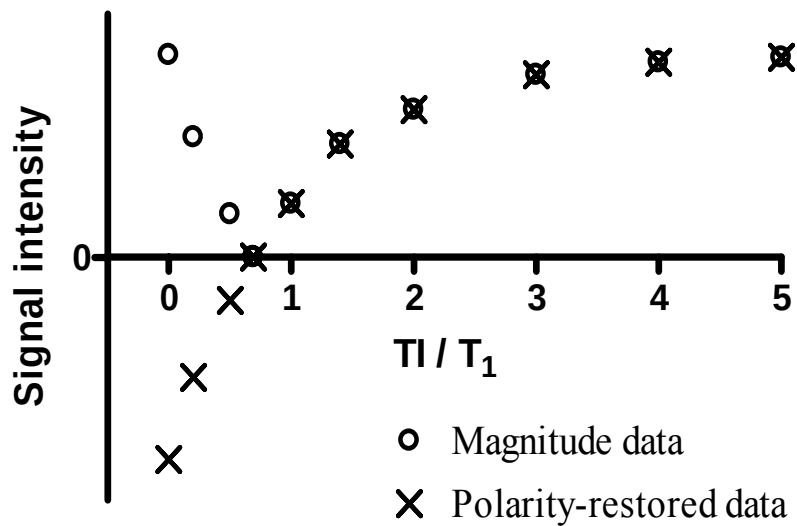


Figure 1-6. Magnitude and polarity-restored data obtained by the inversion recovery sequence.

Saturation recovery method

To measure T_1 by saturation recovery, the sequence comprises of a repeated series of 90° pulses to flip the magnetization into the x-y plane followed by a period of time (TR)

to allow recovery of the longitudinal magnetization. The saturation recovery method is performed over a range of TR values and fitting the obtained SI at different TRs to the equation $SI(TR) = A \times (1 - e^{-TR/T_1})$ to calculate T_1 values.

Magnetization prepared rapid gradient echo method

The equations to fit the MP-RAGE SI data to the signal recovery curve for estimation of T_1 values are complex (Deichmann *et al.*, 2000; Jivan *et al.*, 1997; Kingsley, 1999; Wright *et al.*, 2008). However, the complex equations for fitting MP-RAGE data can be approximated to the same as the IR equation for T_1 measurement when low flip angle and the overall image-repetition time (TR_0) $> 5 T_1$ are employed (Jivan *et al.*, 1997). In appreciation of the approximation made for the calculation of the T_1 value from MP-RAGE data, the T_1 calculated is named T_1 -effective.

1.7 Analysis of tissue manganese (Mn)

Each element consists of electrons, neutrons and protons. Protons and neutrons are found in the nucleus and electrons are located around the nucleus. Following excitation, atoms and molecules absorb and emit electromagnetic radiation at characteristic wavelengths (spectral lines). Atomic absorption spectrometry (AAS) is used to determine elemental composition by measuring the characteristic absorption of radiation passing through a sample (Atkins and De Paula, 2006a). Conversely, emission spectrometry techniques, such as x-ray fluorescence spectrometry (XRF, see section 1.7.1), inductively coupled plasma atomic emission spectrometry (ICP-AES, see 1.7.2) and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) measures the characteristic radiation emitted by a sample following excitation (Atkins and De Paula, 2006b). Emission spectrometry has a lower detection limit of detection than AAA, but is more costly (Hill *et al.*, 2002). These analytical methods measure the total Mn levels in samples and do not distinguish between the different oxidation states of Mn (Barceloux, 1999). In addition, it is worth noting that cross contamination during sample preparation and handling may be an error source during analysis of Mn-containing biological samples (Bolann *et al.*, 2007).

1.7.1 X-ray fluorescence (XRF)

X-ray is a type of electromagnetic wave with extremely short wavelength. After an external excitation by x-ray, an electron is ejected from the inner shell of the atom, creating a vacancy. The vacancy is then filled by an electron from outer shell which loses energy corresponding to the difference in the energy levels involved. The energy

can be released in the form of an x-ray or transferred to another electron. XRF is based on measuring the fluorescence emitted from the elements in a sample following irradiation by x-rays (Pyle, 1996). While each element reveals its unique x-ray spectrum during this process, the acquired x-ray spectrum can be used to identify the unknown elements in a sample. It is a non-destructive analytical method and has been used to measure lead in bone *in vivo* (Todd and Chettle, 1994).

1.7.2 Inductively coupled plasma atomic emission spectroscopy (ICP-AES)

ICP-AES has been used to measure the metal content in biological samples (Frame and Uzgiris, 1998; Zhang *et al.*, 2004). ICP-AES analysis is performed in solution samples and biological samples are usually digested with acids to get the metal into solutions. The resultant solution is then aerolised and atomised and exposed to high energy plasma with production of charged ions (Jarvis and Jarvis, 1992). The sample is excited by plasma, ionised gas, leading to electrons transiting to a higher energy state. As the electrons return to their previous low energy level, they emit electromagnetic radiation at a characteristic set of wavelengths. The wavelength of the emitted radiation depends on the difference between the excited energy level and the ground state. As each element has a unique set of energy levels, each element has a characteristic set of spectral lines. Finally, the optical signals emitted from the elements pass to a light detector and signals within the selected range of wavelengths are converted into electrical signals.

Spectral line overlap is a problem by ICP-AES. To avoid spectral interference, an analytical line is chosen to avoid overlapping by another element in the sample. Thus, the specific wavelengths in the spectrum indicate the elemental composition in a sample and the magnitude of the spectral signal provides the concentrations of the observed elements. The typical wavelength line used for Mn measurement is 257.610 nm. The sensitivity of ICP-AES method is insufficient for extremely low elemental levels and very small sample sizes (Frame and Uzgirish, 1998).

1.7.3 Laser ablation inductively coupled plasma mass spectroscopy (LA-ICP-MS)

Laser radiation may cause ablation, vaporization and excitation of solid samples and therefore a sample preparation method for elemental analysis (Arrowsmith, 1987). LA-ICP-MS uses a laser beam to scan and vaporize solid samples to the vapour phase for mass spectroscopy analysis. The scanning technique makes LA-ICP-MS a method for mapping the spatial distribution of elements with micron-level resolution (Becker, 2002). Working with solution samples, it is easy to obtain suitable calibration and internal standardization for elemental analytic techniques (Arrowsmith, 1987). Therefore, the disadvantage of LA-ICP-MS method is the lack of internal standards and therefore it is difficult to obtain quantitative information (Arrowsmith, 1987; Becker, 2002).

1.8 Hypothesis and aim

The overall aim of this project is to develop and implement MRI techniques to assess location, quantity and function of β -cells *in vivo*. Specific hypotheses and aims for individual studies are listed below.

1.8.1 Mouse pancreatic MRI

The mouse pancreas is a thin and membrane-like organ spreading throughout the upper abdomen. Therefore, delineating and imaging the mouse pancreas *in vivo* by MRI is challenging.

- To gain experience in discerning the pancreas from surrounding organs by MRI with the aid of gross anatomy knowledge
- To explore and optimize MRI methodology for pancreatic imaging *in vivo*.

1.8.2 Validation of the quantitative MP-RAGE method *in vitro*

The MP-RAGE sequence is suitable for *in vivo* pancreatic imaging as it provides improved tissue contrast and is less susceptible to motion. However, MP-RAGE MRI is not well-established for quantitative MRI assessment. Therefore, the ability of MP-RAGE MRI as a quantitative measurement tool for $[\text{Mn}^{2+}]$ was tested in this section.

- To establish the dose-dependent relationship between MnCl_2 concentration and T_1/R_1 values as measured by MP-RAGE MRI
- To test the agreement between T_1 values assessed by IR-SE and MP-RAGE

1.8.3 Pharmacokinetics of MnCl_2 *in vivo*

The purpose of this study was to assess dose-dependent tissue Mn^{2+} accumulation *in vivo* by MRI.

- To establish the dose-dependent relationship between infused MnCl_2 dose and tissue $[\text{Mn}^{2+}]$ by MP-RAGE MRI
- To validate the MRI results by ICP-AES, non-MRI method,
- To establish the tissue Mn^{2+} washout over time

1.8.4 Glucose challenge experiments *in vivo*

Mn^{2+} can act as a surrogate of Ca^{2+} as well as being a positive T_1 contrast agent. These properties make MEMRI a quantitative method for assessing Ca^{2+} -related cellular activities in excitable cells such as neurons and myocytes. Glucose-stimulation of β -cells leads to an intracellular influx of Ca^{2+} and insulin secretion. Therefore, the glucose-stimulated β -cells activity may be detected by MEMRI. Here, the ability of MEMRI as an *in vivo* method to assess β -cell function was tested.

- To assess activation of the functional β -cell mass in response to glucose stimulation in healthy mice by MEMRI
- To identify a reduction of the functional β -cell mass in a diabetic mouse model by MEMRI
- To correlate the Mn distribution measured by non-MRI methods, LA-ICP-MS and XRF, with islet distribution assessed histologically
- To assess the effects of Mn^{2+} and STZ on pancreatic islets by histology and immunohistochemistry

- To apply MEMRI techniques to image Ca^{2+} in other insulin-target organs

2 *Materials and methods*

2.1 Materials

2.1.1 Animals

Male C57BL/6 mice weighted 30g were obtained from Harlan UK (Bicester, Oxon, UK). Animal studies were performed in accordance with the Animals (Scientific Procedures) Act 1986 (UK) under project licence numbers 70/6532 and 70/6656. All mice were housed in individually ventilated cages with 12 h of light and dark cycled (light on at 7 am and off at 7 pm) and with free access to standard chow (RM3, Special Diets Services Ltd, Essex, UK) and drinking water unless otherwise stated. Shortage of animal supply occurred during this work and thus C57BL/6 mice weighted 23 g were ordered for the experiments after the pilot studies.

2.1.2 Reagents

Isoflurane was purchased from Baxter Healthcare Ltd. (Norfolk, UK). Glucose solution (50 % w/v) was obtained from Hameln Pharmaceuticals Ltd (Gloucester, UK). Sterile water was purchased from B. Braun Melsungen AG (Melsungen, Germany). Histoclear was purchased from Raymond A Lamb (East Sussex, UK). Absolute ethanol was obtained from Fisher Scientific Ltd. (Leicestershire, UK). Aristar nitric acid and concentrated Aristar perchloric acid were purchased from BDH (Poole, Dorset, UK).

The following reagents were obtained from Sigma-Aldrich Co Ltd (Poole, Dorset, UK): molecular biology grade 1 M manganese chloride solution (manganese chloride

tetrahydrate, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), streptozotocin powder (STZ, $\text{C}_8\text{H}_{15}\text{N}_3\text{O}_7$), 10 % neutral buffered formalin, hematoxylin and eosin Y (H&E). (Note: STZ and formalin are carcinogens according to the Material Safety Data Sheet and were handled in a chemical fume hood.)

The following reagents were obtained from Dako (Ely, Cambridgeshire, UK) for immunohistochemistry staining: guinea pig anti-insulin antibody (Dako A0561), biotinylated anti-goat/mouse/rabbit immunoglobulin (Dako E0453), streptavidin-biotin complex conjugated with horseradish peroxidase (Dako K0377), diaminobenzidine (DAB) substrate chromogen solution (Dako K4011), rabbit anti-glucagon antibody (Dako A0565) and biotinylated sheep anti-rabbit/goat/mouse immunoglobulin (Dako E0453).

2.2 Equipment

2.2.1 MRI equipment

All MRI experiments were performed on a 4.7T VMRIS scanner (Varian Inc., Palo Alto, USA) and equipped with an actively shielded gradient coil (Magnex Scientific, Oxford, UK) with a maximal gradient strength of 40 G/cm and a rise time of 200 ms based at the Biological Imaging Centre (Clinical Sciences Centre, Imperial College London, UK). For MRI, a quadrature volume coil (35mm internal diameter, Magnetic Resonance Laboratories, Oxford, UK) was used. A plastic bed (home-built) was used to hold the animal, physiological monitoring equipment and infusion apparatus.

2.2.2 Physiological monitoring

Physiological parameters, respiration and rectal temperature, were monitored using Model 1025 Monitoring and Gating System (Small Animal Instruments, Inc., NY, USA) during scanning. Body core temperature was maintained using warm air with an automated temperature control system integrated with the physiological monitoring equipment.

2.2.3 Animal dosing

1 mL syringes and 27G needles were obtained from Becton Dickinson SA (Madrid, Spain; Drogheda, Ireland). 27G butterfly needles and 24G plastic catheters were purchased from Hospira Inc. (Sligo, Ireland; Donegal Town, Ireland). Polyethylene tubing (inner diameter = 0.38 mm, outer diameter = 1.09 mm) was obtained from Portex Ltd (Kent, UK). For i.v. infusions, a PHD 2000 Syringe Pump (Harvard Apparatus, Holliston, MA, USA) was used and positioned outside the magnet. A 2.5 m long polyethylene tube was used to connect the syringe at the syringe pump to the butterfly needle implanted in the tail of the animal. For bolus i.p. injection of glucose or vehicle solutions during scanning, a 2 m long polyethylene tube was used to connect the i.p. catheter implanted in the peritoneal cavity of animals with the syringe located outside the magnet bore.

2.2.4 Blood glucose measurement

The MediSense Optium Xceed Blood Glucose Meter (MediSense, Abbott Laboratories, Ireland) with Optium Plus test strips (Abbott Diabetes Care, Maidenhead, UK) was used to measure blood glucose concentrations.

2.2.5 Analysis of manganese

All the samples for analysis of Mn were prepared in the Biological Imaging Centre (Clinical Sciences Centre). Bulk tissue Mn analysis was performed by ICP-AES using SPECTRO CIROS VISION ICP spectrometer (SPECTRO Analytical Instruments, Inc., MA, USA) at the Centre for Analytic Sciences of Sheffield University in collaboration with Prof. Cameron McLeod. LA-ICP-MS was performed by using an HP-4500 (Agilent Technologies, Manchester, UK) inductively coupled plasma quadrupole mass spectrometer coupled to an Nd:YAG laser ablation system (UP-266 Macro LA system, New Wave Research, Huntingdon, UK). XRF was performed on a Fluo X-ray fluorescence microprobe beamline at the ANKA synchrotron radiation facility (Karlsruhe, Germany) by Dr Po-Wah So. A 4 μm SPEX SamplePrep aluminium film was obtained from Ultralene[®] (New Jersey, U.S.A.) for XRF study.

2.3 Methods

2.3.1 Gross anatomy of the mouse pancreas

Pancreatic imaging in mice is challenging since the organ is a diffuse, but not a well-defined solid organ. Therefore, it is important to gain some experience in the anatomical features of the mouse pancreas prior to the MRI experiments. Examination of the pancreas through dissection of mouse cadavers may assist discernment of the mouse pancreas from the surrounding organs in MR images. Therefore, the gross anatomy of the mouse pancreas was correlated with MRI data.

A male C57BL/6 mouse (~ 30 g) was fasted overnight (~ 18 h) by removal of food at 5 pm prior to the day of scanning, but had free access to drinking water. The animal was killed by neck dislocation after the overnight fast and the abdominal cavity exposed. The anatomical relationship of the pancreas was examined and the photographs of the whole mouse were taken.

2.3.2 Optimization of MRI

2.3.2.1 Introduction

The optimal pulse sequences for rodent pancreatic MRI are: short scan time to avoid motion artefacts, use of thin imaging slices to avoid partial volume averaging and high tissue contrast to visualize the pancreas from the surrounding tissues (see 1.6.3.1). Here, images were acquired with SE and MP-RAGE MRI sequences. The former is the most commonly used MRI pulse sequence and the latter is a 3D T₁-weighted gradient

echo sequence, commonly used for abdominal imaging (Mugler and Brookeman, 1990b). To minimize the effect of circadian variations in insulin secretion, all experiments were performed in the early light phase of the diurnal cycle.

2.3.2.2 Spin echo

2.3.2.2.1 Mouse cadaver imaging

Male C57BL/6 mouse was fasted overnight (~ 18 h) by removal of food at 5 pm prior to the day of scanning but had free access to drinking water (n = 1, 21.6 g). The animal was killed by neck dislocation after the overnight fast and then placed in a plastic bed in a supine position with the mouse at the centre of the RF coil for MRI. MR images were performed with a standard SE sequence with different values of TR and TE. Imaging parameters were as follows: TR = 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1200 ms; TE = 15, 20 and 30 ms; matrix size = 256×128 ; field of view (FOV) = 100×50 mm, average = 2, 10 slices, 1 mm thickness and 0.5 mm gap.

Regions of interest (ROIs) were manually placed by the same experienced operator in the splenic portion of the pancreas (the thickest part of the mouse pancreas), left renal cortex, left liver, left thigh muscle, spleen, myocardium, interscapular and epididymal fat (Figure 2-1). Each ROI was carefully selected in the centre of the organ to avoid partial volume effects and large vessels. ROIs for each organ were of the same size and location throughout the experiment within the same animal, but varied between individual animals. Areas of ROIs were $\sim 2\text{mm}^2$ in the spleen, kidney and BAT; $\sim 3\text{mm}^2$ in the pancreas, WAT and heart; $\sim 5\text{mm}^2$ in the liver and muscle. Analysis of

ROI was carried out using Image J software (Image J 1.3.1, NIH, USA). Differences in SI between the pancreas and its adjacent organs – the liver, left kidney and spleen, were calculated. It is worth noting that the ROIs were selected from a part but not the entirety of an organ of interest, and a non-experienced operator is likely to have difficulties in placing ROIs in the pancreas and adipose tissue.

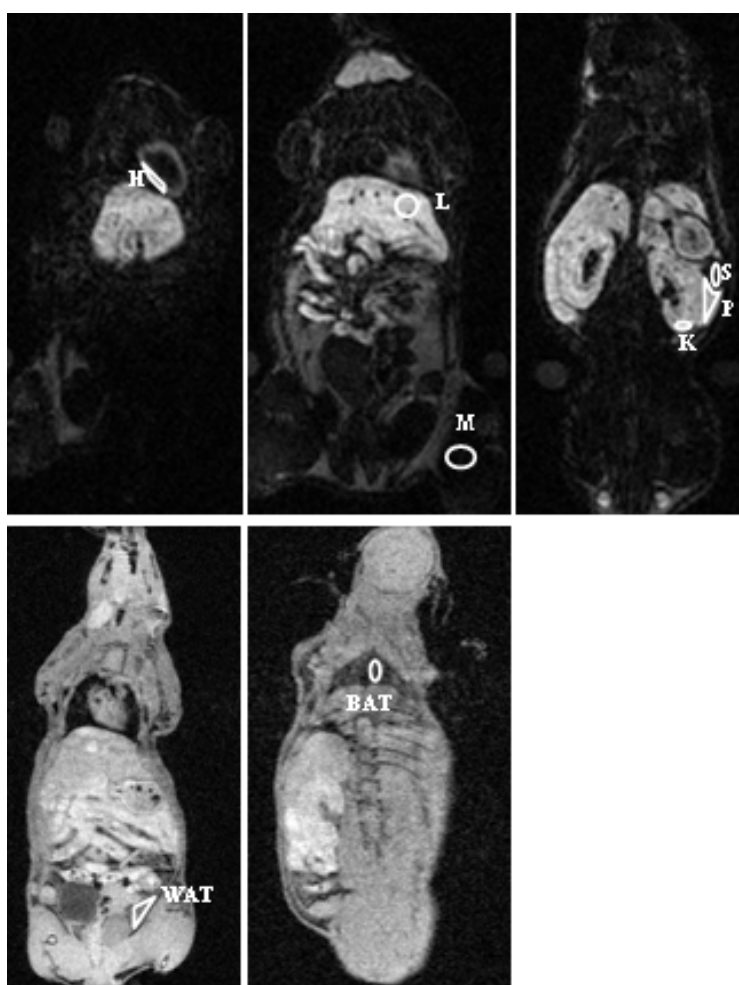


Figure 2-1. Coronal MP-RAGE MRI images showing placement of regions of interest in the heart (H), left kidney (K), left liver (L), left thigh muscle (M), gastrosplenic portion of the pancreas (P), spleen (S), epididymal fat pad (white adipose tissue, WAT) and interscapular fat pad (brown adipose tissue, BAT) in a C57BL/6 mouse after 36 min after the start of MnCl_2 infusion (2 mM, 0.1 mL, 0.2 mL/h).

2.3.2.2.2 Anesthetized mice imaging

Physiological motion resulting from respiration, the beating heart, and bowel peristalsis during abdominal MR scan can cause blurring and ghosting, leading to degradation of image quality. Image quality can be improved by respiratory gating which suppresses motion artefacts in abdominal MRI. In this study, respiratory movement was detected using a pneumatic pillow fixed to the abdomen of the mouse. In respiratory gating, the inspiration wave was utilized to trigger the imaging sequence so that images were acquired during expiration which is the relatively still phase of the respiratory cycle.

Male C57BL/6 mouse was fasted overnight ($n = 1$, ~ 23 g before fasting) and anaesthesia induced with a mixture of 2 L/min oxygen and 4 % isoflurane, and maintained during scanning with 1 L/min oxygen and 1-2 % isoflurane via a nose cone. Scans were performed with the optimal parameters obtained previously in the mouse cadaver experiment (see section 2.3.2.2.1): TR = 900 ms, TE = 15 ms, matrix size = 256×128 ; FOV = 100×50 mm, averages = 2, 10 slices, 1 mm thickness and 0.5 mm gap. During MR scanning, respiration and rectal temperature were continuously monitored. Core temperature was maintained by a warm air heating system with automated feedback control system at a set point of 37°C. ROIs were selected from the pancreas, kidney, liver and spleen as described previously (see section 2.3.2.2.1) and SI measured. SI differences between the pancreas and its adjacent organs – the liver, left kidney and spleen, were calculated.

The same animal was then scanned with the same parameters, but with respiratory gating. Since the acquisition was triggered by the inspiration wave, TR in SE images

with respiratory gating was dependent on the respiratory rate. To achieve a constant TR of 900 ms, the depth of anaesthesia was controlled to maintain a respiratory rate of 67 beats/min.

2.3.2.3 MP-RAGE

The MP-RAGE sequence was modified from a standard 3D gradient-echo sequence by incorporating a magnetization preparation pulse and a data acquisition loop.

2.3.2.3.1 TI

As mentioned previously (see section 1.6.3.4.2), the value of TI chosen needs to be greater than the $T_{I_{null}}$ of tissues to obtain positive SI data. Further, the variation of SI or slope is the greatest around the null point. Therefore, the optimal TI needs to be set to be greater than the null point of the tissue with the longest T_1 . To detect the optimal TI value, different TI values were used in the MP-RAGE experiment.

Male C57BL/6 mice were fasted overnight ($n = 2$, 21.6 ± 0.2 g) and anaesthetized as described previously (see section 2.3.2.2.2). Imaging was performed without $MnCl_2$ infusion with parameters: TI = 10, 16, 27, 45, 73, 120, 200, 330, 540, 890, 1460, 2400 ms; flip angles = 10° ; TR = 10 ms; TE = 2.4 ms; TD = 2 s; 1 average, matrix size = $256 \times 128 \times 64$ and FOV = $100 \times 50 \times 32$ mm. ROIs were selected from the pancreas, kidney, liver, skeletal muscle, heart, spleen and epididymal fat as described previously (see section 2.3.2.2.1) and SI measured. Then, polarity of the SI data was restored

manually and fitted to the equation: $SI(TI) = A - B \times e^{-TI/T_1}$ (see equation 1 in section 1.6.3.4.2) using PRISM 4 (GraphPad Software, Inc., San Diego, CA, USA) where 100 % longitudinal magnetization recovery was not assumed.

2.3.2.3.2 Flip angle

An optimal flip angle gives sufficient tissue contrast between the pancreas and surrounding organs for the delineation of the mouse pancreas. Therefore, the SI in tissues at different flip angles was measured and the difference in SI between the mouse pancreas and surrounding organs including the kidney, liver and spleen was also calculated.

Male C57BL/6 mouse was fasted overnight ($n = 1$, ~ 23 g before overnight fasting) and anaesthetized as described previously (see section 2.3.2.2.2). $MnCl_2$ solutions were prepared by dilution of the 1 M $MnCl_2$ solution with different amount of sterile water. A 27 gauge butterfly cannula was inserted into the tail vein. Infusion of the $MnCl_2$ solution was performed using a syringe pump for delivery of $MnCl_2$ solutions,

MP-RAGE images were acquired prior to and 10-60 min after the end of $MnCl_2$ infusion (20 mM, 0.2 mL/h, 0.1 mL, taking 30 min) in the same animal with the same parameters. Imaging was performed before and after $MnCl_2$ infusion with parameters: flip angles = 5°, 10°, 15°, 20°, 25°, 30°, 35°, 40°, 45° or 50°; TR = 10 ms; TE = 2.4 ms; delay time (TD) = 2 s; TI = 740 ms; 1 average, matrix size = $256 \times 128 \times 64$; and FOV = $80 \times 40 \times 20$ mm. ROIs were selected from the pancreas, kidney, liver and spleen as

described previously (see section 2.3.2.2.1) and SI measured. SI differences between the pancreas and its adjacent organs – the liver, left kidney and spleen were calculated. The same analysis was performed for both with and without MnCl_2 infusion images.

2.3.3 Validation of MP-RAGE T_1 measurement

Phantom studies were conducted to test agreement of T_1 measurements by MP-RAGE and standard inversion recovery spin-echo (IR-SE) methods, the latter being the conventional method of measuring T_1 (see section 1.6.3.4.2). Both methods were performed with TR (or TD) = 15 s ($\gg 5 \times T_1$ of water) to allow full recovery of longitudinal magnetization. A MP-RAGE method with shorter scan time, 5 TIs and TD = 2 s, was also applied for comparison. MnCl_2 solutions were prepared by dilution of the 1 M MnCl_2 solution with different amounts of sterile water. Tubes containing MnCl_2 solutions at concentrations of 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2, 0.22, 0.24, 0.3, 0.5 and 1 mM were placed in a sample holder for MRI.

2.3.3.1 Inversion recovery spin-echo (IR-SE)

T_1 measurements were made using IR-SE with the follow parameters: TI = 20, 40, 60, 80, 100, 140, 200, 300, 400, 500, 700, 900, 1200, 1500, 2000, 2500, 3000 and 4500 ms; TR = 15 s; TE = 11.97 ms; TD = 2000 ms; matrix size = 256×128 ; FOV = 90×45 mm; 2 averages; 2 mm thickness and a single slice. The accuracy of the T_1 measurement was improved by performing the experiment fully T_1 -relaxed with TR set at 15 s. Total acquisition time for all 18 images was 18 h.

2.3.3.2 MP-RAGE

Imaging parameters for T_1 measurement were as follows: $TI = 100, 200, 500, 1000$ and 2400 ms; $TR = 10$ ms; $TE = 2.4$ ms; $TD = 2$ s; flip angle = 10° ; 1 scan, matrix size = $256 \times 128 \times 64$; $FOV = 80 \times 40 \times 20$ mm with a total scan time of ~ 22 min. T_1 measurement was also performed with the same parameters except $TD = 15$ s and $TI = 20, 40, 60, 80, 100, 140, 200, 300, 400, 500, 700, 900, 1200, 1500, 2000, 2500, 3000$ and 4500 ms with a total scan time of ~ 18 h.

2.3.3.3 Imaging and data analysis

To avoid partial volume effects, ROIs were placed in the centre for each tube. ROIs were of the same size for different tubes and placed at the same position for all scans using Image J (NIH, USA). Then, the SI data were measured from the ROIs, polarity of the modulus data was restored manually.

For T_1 estimation, MRI data by both IR-SE and MP-RAGE methods were fitted to the usual 3 parameter fit IR equation: $SI(TI) = A - B \times e^{-TI/T_1}$ (see equation 1 in section 1.6.3.4.2) using PRISM 4, where 100 % longitudinal magnetization recovery was not assumed. The measured T_1 values by MP-RAGE MRI method were defined as T_1 -effective values. A scatter plot was made using PRISM 4 to investigate the relationship between T_1 -effective and T_1 data obtained by IR-SE and MP-RAGE methods, respectively. A Bland-Altman plot was also displayed using PRISM 4 to compare T_1 measurement methods by IR-SE and MP-RAGE.

2.3.4 Dose-dependent effects of MnCl_2

The effects of different MnCl_2 doses on SI and T_1 values measured by *in vivo* MP-RAGE MRI were established. Further, the T_1 values by *in vivo* MP-RAGE MRI data was correlated with *in vitro* tissue [Mn] analysis by ICP-AES. The objective was to determine the appropriate MnCl_2 dose for MEMRI of the pancreas. The dose needs to be as low as possible to avoid the potentially toxic effects of MnCl_2 infusion, while still being capable of enhancing the activated pancreas, so that it is distinguishable from the non-activated pancreas. Imaging was performed in a serial manner to observe the change in enhancement in the pancreas with MnCl_2 infusion. A static T_1 measurement was also made at 36 mins after the start of MnCl_2 infusion.

2.3.4.1 Serial MEMRI

2.3.4.1.1 Animal preparation

Fifteen male C57BL/6 mice (19.7 ± 0.4 g) were fasted overnight and prepared for MRI as detailed previously (see section 2.3.2.2.2). Briefly, animals were anaesthetized and a cannula was inserted into the tail vein for infusion of MnCl_2 at concentrations of 2, 5, 10, 20 or 40 mM ($n = 3/\text{group}$) using a syringe pump (0.2 mL/h, 0.1 mL final volume) for 30 min. The highest MnCl_2 dose (40 mM, 0.1 mL) employed in this study is equivalent to the LD_{50} for C57BL/6 mice, 38 mg/kg body weight (Silva *et al.*, 2004).

2.3.4.1.2 MRI measurement

MEMRI protocols in this thesis were modified from previous MEMRI studies in our group (Kuo *et al.*, 2005; Kuo *et al.*, 2007). MP-RAGE MRI was performed with the following parameters: TI = 740 ms, TR = 10 ms, TE = 2.4 ms, matrix size = $256 \times 128 \times 64$, FOV = $100 \times 50 \times 32$ mm, flip angle = 10° , TD = 2 s, 1 scan and acquisition time was ~ 4 min. Three datasets were collected prior to MnCl_2 infusion and 24 datasets during and after the end of infusion according to Figure 2-2. Data was collected for 96 min after the start of infusion.

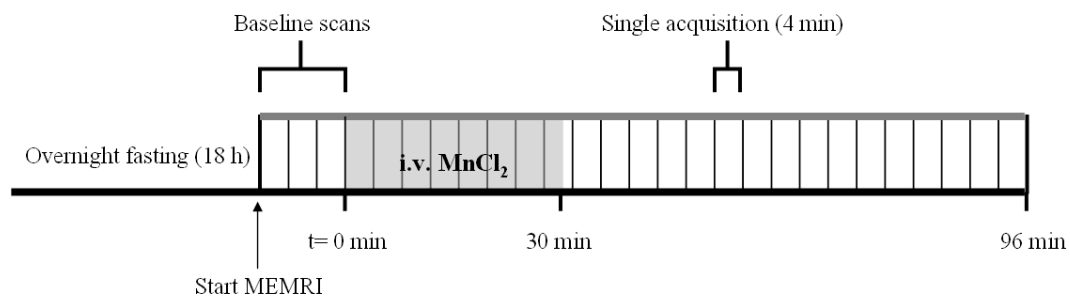


Figure 2-2. Schematic of MEMRI protocol for determining dose-dependent SI changes over time. Prior to MRI, C57BL/6 mice were fasted overnight. After three baseline acquisitions, MnCl_2 was given intravenously (i.v.) for a period of 30 min (2 mM, 0.1 mL, 0.2 mL/h). Data were continuously recorded and 27 datasets acquired.

2.3.4.1.3 Image analysis

ROIs of the pancreas were selected and SI obtained as described previously (see section 2.3.2.2.1). The baseline signal intensity (SI_0) is the average SI of three MRI datasets

acquired before the start of MnCl_2 infusion and SI_t is the SI at time t (Kuo *et al.*, 2005).

The serial MEMRI data was presented as the percentage change in SI from baseline:

$$\% \text{ SI change} = (\text{SI}_t - \text{SI}_0) / \text{SI}_0 \times 100 \%$$

The steady state signal enhancement was defined as the mean of the percentage changes in SI during $t = 36\text{-}96$ min after the start of MnCl_2 infusion.

2.3.4.2 T_1 measurement

2.3.4.2.1 Animal treatment

A total of 26 male C57BL/6 mice (21.7 ± 0.2 g after fasting) were fasted overnight and animals prepared for MRI as detailed previously (see section 2.3.2.2.2), except six mice did not have a cannula implanted in the tail. The remaining animals were divided into 5 groups ($n = 4/\text{group}$) and each group received MnCl_2 at concentrations of 2, 5, 10, 20 or 40 mM at a rate of 0.2 mL/h and a total infused volume of 0.1 mL for 30 min. There was no significant difference in body weight between all the groups.

2.3.4.2.2 MRI measurement

T_1 measurements by MP-RAGE MRI were performed 6 min after the end of MnCl_2 infusion (or 36 min after induction of anaesthesia in non- MnCl_2 -treated animals) as in Figure 2-3. The following parameters were used: TI = 100, 200, 500, 1000 and 2400 ms; TR = 10 ms; TE = 2.4 ms, matrix size = $256 \times 128 \times 64$, FOV = $80 \times 40 \times 20$ mm, flip angle = 10° , TD = 2 s, 1 scan and total acquisition time was ~ 22 min (as in *in vitro*

study). A smaller FOV than that in serial MEMRI was used to allow isotropic resolution due to the upgrade of MRI software and improvement of animal bed.

2.3.4.2.3 Image analysis

SI data were measured from ROIs placed on the pancreas, kidney, liver, heart, spleen, muscle, epididymal fat and interscapular fat as described previously (see section 2.3.2.2.1). T_1 -effective was calculated as detailed in section 2.3.3.3.

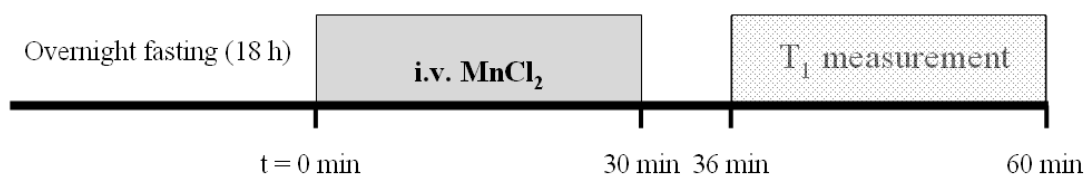


Figure 2-3. Schematic of T_1 -effective measurement protocol by MP-RAGE. Prior to MRI, C57BL/6 mice are fasted overnight. $MnCl_2$ is given intravenously (i.v.) for a period of 30 min. The T_1 -effective measurement was obtained from MRI data acquired 6 min after the end of $MnCl_2$ infusion.

2.3.4.3 Correlation of tissue R_1 -effective and tissue [Mn]

2.3.4.3.1 Animal treatment

A total of 18 male C57BL/6 mice (22.0 ± 0.2 g after fasting) were fasted overnight and animals prepared for MRI as detailed previously (see section 2.3.2.2.2), except three mice did not have a cannula implanted in the tail. 15 of the animals were divided into

groups of three and each group received MnCl_2 at concentrations of 2, 5, 10, 20 or 40 mM at a rate of 0.2 mL/h and a total infused volume of 0.1 mL for 30 min. There was no significant difference in body weight between all the groups. T_1 measurement was performed at 36 min after the start of MnCl_2 infusion. Animals were euthanized by neck dislocation at 66 min after the start of MnCl_2 infusion (see Figure 2-4). Tissues including the pancreas, kidney, liver, skeletal muscle, epididymal fat, spleen and heart were collected, immediately frozen with dry ice and then stored in a -80°C freezer prior to ICP-AES.

2.3.4.3.2 MRI measurements and image analysis

T_1 -effective measurements were performed by MP-RAGE method as before (section 2.3.4.2) and T_1 -effective values were calculated for the pancreas, kidney, liver, heart, spleen, skeletal muscle and epididymal fat as described in section 2.3.4.2. R_1 -effective value was also calculated by taking the reciprocal value of T_1 -effective.

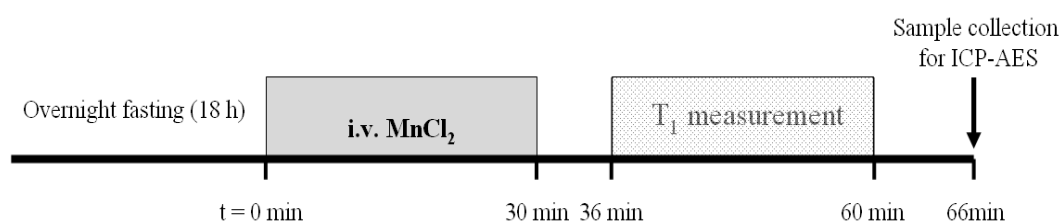


Figure 2-4. Schematic of T_1 -effective measurement and tissue sampling. Prior to MRI, C57BL/6 mice were fasted overnight and MnCl_2 given intravenously (i.v.) for a period of 30 min. T_1 -effective measurements were acquired 36-60 min after the start of MnCl_2 infusion. After scanning, animals were euthanized and tissues collected 66 min after the end of MnCl_2 infusion.

2.3.4.3.3 ICP-AES

Tissues were taken from storage and dried for 60 h at 110°C ($n = 3/\text{dose}$). The dried mass was weighed and digested with 3 ml of concentrated nitric acid and 0.5 mL of concentrated perchloric acid for 18 h in a fume-hood. For complete solubilisation, the acidified sample was heated as follows: 1 h at 120°C, 2 h at 150°C, 1 hr at 180°C and 2 h at 200°C. Finally, the resulting solutions (0.5 mL) were diluted to a final volume of 10 mL with 1 % nitric acid for analysis.

Tissue [Mn] was determined by ICP-AES using a SPECTRO CIROS VISION ICP spectrometer. The Mn emission line Mn^{2+} at 257.611 nm was used as the analysis wavelength. The [Mn] in solution ($\mu\text{g/mL}$) was converted into $\mu\text{g/g}$ using the dry-mass and solution volume of each sample.

2.3.5 Tissue manganese washout

The pharmacokinetic profile of Mn^{2+} after a systemic dose is essential for designing MEMRI protocols. However, so far there is no such *in vivo* information about the accumulation and washout of Mn^{2+} in mouse abdominal organs. To better understand its pharmacokinetic properties, wash-in and wash-out curves of Mn^{2+} in different organs were established by measuring T_1 -effective over time following MnCl_2 infusion using MP-RAGE MRI.

2.3.5.1 Animal preparation

Male C57BL/6 mice were fasted overnight ($n = 3$, 21.2 ± 0.3 g) and the animals prepared for MRI as detailed previously (see section 2.3.2.2.2). A single dose of MnCl_2 (2 mM, 0.2 mL/h, 0.1 mL) was infused through the i.v. cannula for 30 min. Animals were scanned 24 h prior to and 1, 4, 24, 48 and 120 h after the start of MnCl_2 infusion (Figure 2-5). Mice were recovered from anaesthesia and had free access to standard chow between scanning. Anaesthesia was induced again for subsequent scanning.

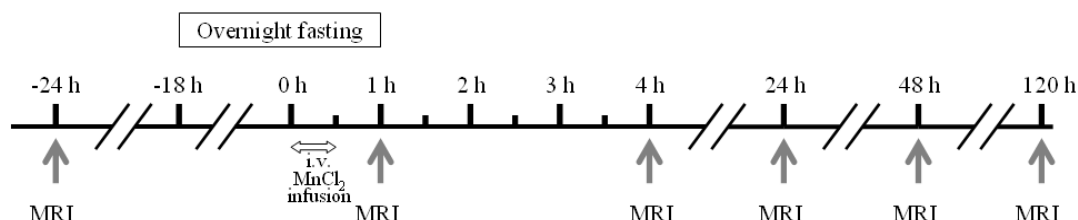


Figure 2-5. Schematic of MEMRI protocol for measuring temporal changes in tissue Mn^{2+} accumulation. The start of MnCl_2 infusion was $t = 0$. Serial MP-RAGE MRI images were acquired 24 h prior to (-24 h) and at 1, 4, 24, 48 and 120 h after the start of MnCl_2 infusion. Animals have no access to food from 18 h prior to MRI scanning.

2.3.5.2 MRI measurement and image analysis

MP-RAGE sequence was performed with parameters: $\text{TI} = 100, 200, 300, 400, 500, 600, 700, 800, 1200$ and 3000 ms; $\text{TR} = 10$ ms; $\text{TE} = 2.4$ ms; $\text{TD} = 3$ s; flip angle = 10° ; 1 average, matrix size = $256 \times 128 \times 64$; $\text{FOV} = 80 \times 40 \times 20$ mm with a total scan time of ~ 60 min. SI data were measured from ROIs placed on the pancreas, kidney, liver and

muscle as described previously (see section 2.3.2.2.1). T_1 -effective values at different time points were calculated as in section 2.3.3.3.

2.3.6 Glucose challenge experiments

2.3.6.1 Healthy mice

Intraperitoneal injection of 2 g/kg body weight dose of glucose has been shown to trigger β -cell insulin secretion in rodents. Therefore, the effect of glucose or water (vehicle, control) on changes in MEMRI SI over time and T_1 -effective values were monitored to determine whether either measurement type was capable of determining differences in β -cell activity *in vivo*. Also, the effect of $MnCl_2$ on glucose levels was measured. Further, MEMRI and T_1 measurement were also investigated in insulin targeted organs: skeletal muscle, liver and adipose tissues.

2.3.6.1.1 Serial MEMRI

Twelve C57BL/6 mice were fasted overnight and anaesthetized. Animals were prepared for MRI as detailed in section 2.3.2.2.2. $MnCl_2$ (2 mM, 0.1 mL at 0.2 mL/h) was infused through the tail vein cannula for 30 min. For glucose challenge experiments, a 24 gauge radiopaque catheter was also implanted into the right lower quadrant of the peritoneal cavity for injection of glucose (50 % w/v, 2g/kg body weight, 0.004 mL/g body weight) or sterile water (0.004 mL/g body weight) prior to placement in the magnetic bore for MRI. One group of mice (2 g/kg, $n = 6$, 20.1 ± 0.7 g) received i.p. injection of glucose solution and another group (control, $n = 6$, 21.3 ± 0.4 g)

received the equivalent amount of sterile water (0.004 mL/g body weight) water at 16 min after the start of MnCl_2 infusion as shown in Figure 2-6.

Briefly, three MRI datasets were collected prior to MnCl_2 infusion and 24 datasets during and after the end of infusion. Data was collected for 96 min after the start of infusion. MP-RAGE MRI was acquired with the parameters: $\text{TI} = 740$ ms, $\text{TR} = 10$ ms, $\text{TE} = 2.4$ ms, matrix size = $256 \times 128 \times 64$, $\text{FOV} = 100 \times 50 \times 32$ mm, flip angle = 10° , $\text{TD} = 2$ s, 1 scan and acquisition time was ~ 4 min (as in section 2.3.4.1.2). SI data were measured from ROIs placed on the pancreas, left femur, liver and epididymal fat. Percentage change in SI was calculated as $\% \text{ SI change} = (\text{SI}_t - \text{SI}_0) / \text{SI}_0 \times 100 \%$ (as in section 2.3.4.1.3).

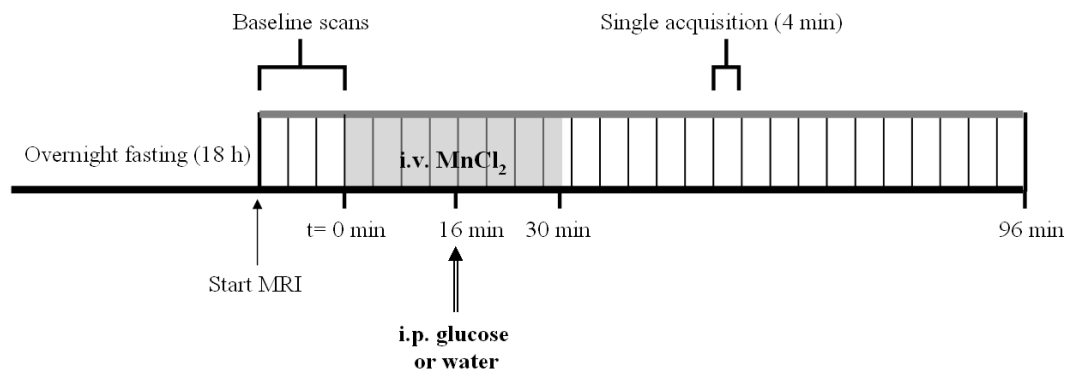


Figure 2-6. Schematic of MEMRI protocol for signal intensity changes over time after glucose or vehicle stimuli. Prior to MRI, C57BL/6 mice are fasted overnight. After three baseline acquisitions, MnCl_2 (2 mM, 0.1 mL, 0.2 mL/h) is given intravenously (i.v.) for a period of 30 min. Bolus i.p. injection of glucose or vehicle was given at 16 min after the start of MnCl_2 infusion. The time course is continued until a total of 27 consecutive images have been acquired.

2.3.6.1.2 T_1 measurement with delay time = 2 s

Overnight-fasted C57BL/6 mice ($n = 15$) were treated with i.v. infusion of MnCl_2 and i.p. injection of either sterile water ($n = 7$, 20.1 ± 0.6 g) or glucose solution ($n = 8$, 20.2 ± 0.8 g). T_1 measurement was performed at 40-63 min after the start of MnCl_2 infusion with the following parameters: $\text{TI} = 100, 200, 500, 1000$ and 2400 ms; $\text{TR} = 10$ ms; $\text{TE} = 2.4$ ms, matrix size = $256 \times 128 \times 64$, $\text{FOV} = 80 \times 40 \times 20$ mm, flip angle = 10° , $\text{TD} = 2$ s, 1 scan and total acquisition time was ~ 22 min (as in *in vitro* study).

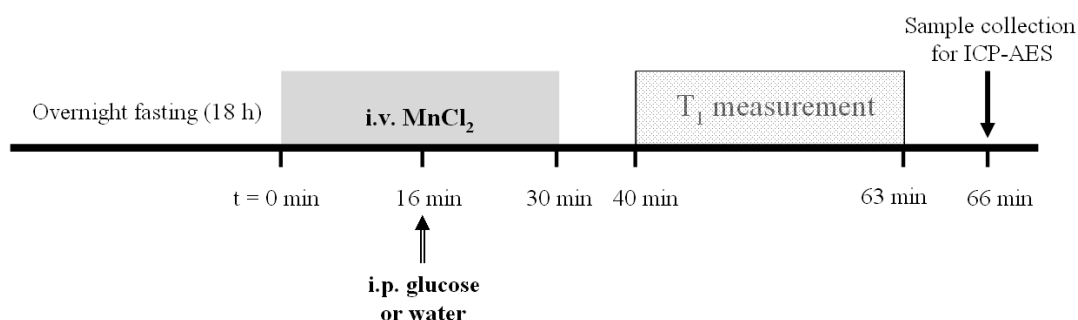


Figure 2-7. Schematic of T_1 -effective measurement protocol. Prior to MRI, C57BL/6 mice were fasted overnight. MnCl_2 is given intravenously (i.v.) for a period of 30 min. Bolus i.p. injection of glucose or vehicle was given at 16 min after the start of MnCl_2 infusion. The T_1 -effective measurement is obtained from MRI data acquired 36-60 min after the start of MnCl_2 infusion.

SI data were measured from ROIs placed on the pancreas, muscle, liver and epididymal fat as described previously (see section 2.3.2.2.1). T_1 -effective values were calculated as in section 2.3.3.3. R_1 -effective value was also calculated by taking the reciprocal

value of T_1 -effective. These T_1 -effective data of the pancreas were also compared with that of non-MnCl₂ treated group in section 2.3.4.2. Animals were euthanized by neck dislocation at 66 min after the start of MnCl₂ infusion. Samples including pancreas, liver, skeletal muscle from left femur and epididymal fat were collected, immediately frozen with dry ice and then stored in a -80°C freezer prior to ICP-AES (see Figure 2-7).

2.3.6.1.3 T_1 measurement with delay time = 3 s

While skeletal muscle has a longer T_1 -effective values compared to other organs, a longer TR_0 is required for recovery of longitudinal magnetization recovery. Therefore, T_1 -effective measurement was also performed in skeletal muscle with a longer TD (= 3 s) to increase TR_0 .

C57BL/6 mice ($n = 12$, 20.9 ± 0.2 g after overnight fasting) were treated as before (see 2.3.6.1.2) and injected i.p. with water or glucose solution ($n = 6$ /group). T_1 -effective values of skeletal muscle was acquired at 50-110 min after the start of MnCl₂ (Figure 2-8). MP-RAGE MRI was performed with parameters: TI = 100, 200, 300, 400, 500, 600, 700, 800, 1200 and 3000 ms; TR = 10 ms; TE = 2.4 ms; TD = 3 s; flip angle = 10°; 1 scan, matrix size = $256 \times 128 \times 64$; FOV = $80 \times 40 \times 20$ mm with a total scan time of ~ 60 min (as in section 2.3.5.2). SI data were measured from ROIs placed on skeletal muscle. T_1 -effective values were calculated as in section 2.3.3.3. R_1 -effective value was also calculated by taking the reciprocal value of T_1 -effective.

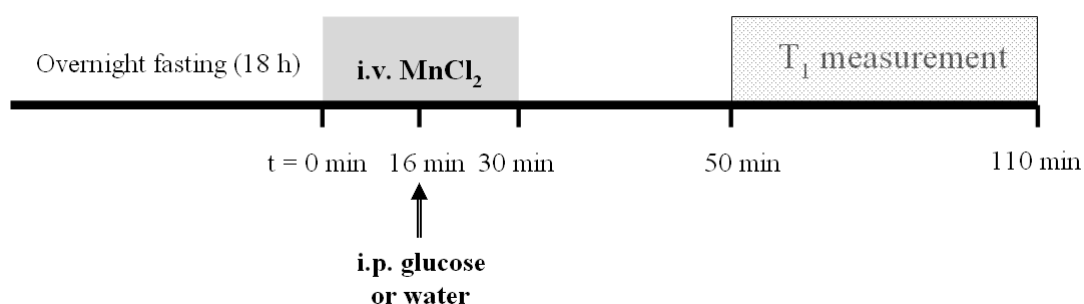


Figure 2-8. Schematic of T₁-effective measurement protocol. Prior to MRI, C57BL/6 mice were fasted overnight. MnCl₂ is given intravenously (i.v.) for a period of 30 min. T₁-effective values were obtained from MRI data acquired 50-110 min after the start of MnCl₂ infusion.

2.3.6.1.4 Tissue [Mn] by ICP-AES

Pancreas, liver, left femoral muscle and epididymal fat from animals in section 2.3.6.1.2 were collected at the end of MEMRI (66 min after the start of MnCl₂ infusion), immediately frozen with dry ice and then stored in a -80°C freezer prior to tissue [Mn] measurement by ICP-AES. ICP-AES was performed as in 2.3.4.3.3.

Unfortunately, some of the tissue [Mn] obtained from left femoral muscle samples as above were below the detection limit of ICP-AES. Therefore, tissue [Mn] in skeletal muscle was reanalysed in samples collected from bilateral femoral muscles to increase the mass of samples. This was done in another batch of animals as below.

Overnight-fasted (18 h) mice received MnCl_2 infusion and i.p. glucose ($n = 10$, 20.4 ± 1.6 g) or water ($n = 10$, 20.3 ± 0.9 g), but not MRI scanning, were killed 66 min after the start of MnCl_2 infusion as above (section 2.3.6.1.2). Additional mice ($n = 4$, 19.8 ± 0.9 g) were used as baseline group without receiving i.p. and i.v. treatment. Bilateral femoral muscles from animals were collected, immediately frozen with dry ice and then stored in a -80°C freezer prior to ICP-AES.

2.3.6.1.5 Tissue [Mn] by LA-ICP-MS: a pilot study

Two C57BL/6 mice (~ 23 g) were fasted overnight for 18 h. Animals received MnCl_2 infusion (20 mM, 0.2 mL/h, 0.1 mL) and i.p. glucose or water were killed 66 min after the start of MnCl_2 infusion as above (section 2.3.6.1.2). Samples including the pancreas and spleen were collected, immediately frozen with dry ice and then stored in a -80°C freezer. Prior to LA-ICP-MS, samples were embedded in OCT media (VWR, UK), and sections were cut at $5\ \mu\text{m}$ using a cryostat.

The laser ablation system was configured to perform multiple parallel line rasters to generate 2D mapping for elements. The laser was operated at a frequency of 10 Hz and a laser power of 1.25 mJ. The scan was performed in a raster pattern at a speed of $60\ \mu\text{m/s}$ with a laser beam diameter of $100\ \mu\text{m}$. The rasters were separated by $200\ \mu\text{m}$ to prevent contamination. Images were produced using Graphis software package (Kylebank Software Ltd, Ayr, UK). The isotopes ^{55}Mn and ^{57}Fe were monitored in a time-resolved mode by ICP-MS. H&E staining was performed on an adjacent section.

2.3.6.1.6 Tissue [Mn] by XRF: a pilot study

Two C57BL/6 mice (~ 23 g) were fasted overnight for 18 h prior to being anaesthetised. Animals received MnCl_2 infusion (2 mM, 0.2 mL/h, 0.1 mL) and i.p. glucose or water (as described in section 2.3.6.1.2). The animals were killed 36 min after the start of MnCl_2 infusion and the pancreas and spleen were harvested and fixed in 10 % neutral buffered formalin for 24-48 h. Then, the samples were processed and embedded in wax blocks. Paraffin-embedded specimens were cut at 5 μm thickness and sections prepared on slides, dried overnight, deparaffinized in xylene and re-hydrated in 100 % and 70 % ethanol for H&E staining to indicate islet location. An adjacent section was mounted onto XRF film for XRF.

For XRF, the beam energy was tuned to 10 eV and focussed to $300 \times 300 \mu\text{m}$ and scanned in a raster fashion. Samples were mounted at a 45° angle with respect to the incoming X-ray beam in the plane of the orbiting electrons to minimize the scatter contribution to the detected signal. The data at each scan point is collected such that each point receives the same amount of incident radiation, and is in the form of a spectrum showing the characteristic radiation from elements in the sample. The spectra are then fitted and peak areas quantified using AXIL (a programme that fits the element K_α and K_β peaks under consideration, taking into account of line overlaps and subtracts the background) to quantify the iron content: 2D maps of iron distribution were constructed using IDL 7.0 (ITT Virtual Information Software).

2.3.6.2 Diabetic mouse model

2.3.6.2.1 Induction of diabetes in a mouse model

For induction of diabetes, male C57BL/6 mice (~ 27.8g) were injected i.p. with a single 180 mg/kg dose of STZ (0.2 mL/mouse). STZ was formulated within 15 min prior to administration and injected at 2 pm without fasting of the animal. Blood glucose and body weight were measured 1 week prior to STZ injection, and at 1 and 3 weeks post-injection. Animals were included in the MEMRI study when a blood glucose level of ≥ 20 mM was measured; approximately 10-17 days after STZ injection.

2.3.6.2.2 Serial MEMRI

Overnight-fasted (18 h) STZ-treated diabetic mice were treated with i.v. infusion of MnCl_2 and i.p. injection of either sterile water ($n = 4$, 24.4 ± 1.0 g) or glucose solution ($n = 4$, 23.9 ± 0.4 g) as for healthy mice (see section 2.3.6.1.1). Serial MEMRI and image analysis were performed as for healthy mice (see section 2.3.6.1.1). SI data were measured from ROIs placed on the pancreas, skeletal muscle and liver.

2.3.6.3 The effect of MnCl_2 on blood glucose levels

In order to determine whether MnCl_2 infusion affected blood glucose levels, we compared the blood glucose changes over time in response to exogenous glucose in control and MnCl_2 -treated mice.

Male C57BL/6 mice ($n = 23$, ~ 23 g) were fasted overnight (18 h) to achieve baseline blood glucose levels. Mice received a tail-vein infusion of MnCl_2 (2 mM, 0.1 mL at 0.2 mL/h) for 30 min. At 16 min after the start of MnCl_2 infusion, an i.p. injection of glucose (2 g/kg) or water ($n = 4$ /group) were given.

A drop of blood was taken from the tail vein by tail vein puncture with a 27 G needle. Blood glucose levels were measured by applying a drop of blood ($> 0.3 \mu\text{L}$) directly to a test strip placed in a blood glucose meter. Samples were collected prior to and 32, 36, 40, 44, 48, 52, 56 and 60 min after the start of MnCl_2 infusion. Blood sampling was not performed during the period of MnCl_2 administration so as to avoid disturbance of Mn delivery.

Measurements were also taken at corresponding times in anaesthetized mice that received an i.p. injection of either glucose or water, but without MnCl_2 infusion ($n = 5$ /group). For control, tail vein blood was also taken in a group of mice ($n = 5$) without any treatment and were also measured at the corresponding times. All the mice were not allowed to recover after the experimental procedures.

2.3.7 Histological analysis of the pancreas

C57BL/6 mice (~ 23 g) were fasted overnight and treated with or without i.v. infusion MnCl_2 (2 mM, 0.1 mL at 0.2 mL/h) for 30 min ($n = 2$ /group). The animals were killed by neck dislocation at 66 min after the start of MnCl_2 infusion and pancreata removed. Additional two STZ-induced diabetic mice (~ 27 g, fed blood glucose ≥ 20 mM)

without treatment were also killed by neck dislocation and pancreata removed. Samples were prepared as describe in section 2.3.6.1.6. Three consecutive sections were obtained from each mouse pancreatic sample for H&E, insulin and glucagon staining. H&E staining was used to assess the distribution and morphology of pancreatic islets.

Insulin staining was used to assess insulin-containing β -cells. Slides prepared above were incubated with guinea pig anti-insulin antibody (1:50) for 90 min at room temperature and then with biotinylated swine anti-goat/mouse/rabbit immunoglobulin (1:200) for 30 minutes. Visualization was achieved by streptavidin-biotin complex conjugated with horseradish peroxidase for 30 minutes followed by diaminobenzidine chromogen solution for 10 min.

Glucagon staining was used to analyse glucagon-containing α -cells. Slides were incubated with rabbit anti-glucagon antibody (1:200) for 60 min at room temperature, with biotinylated sheep anti-rabbit/goat/mouse immunoglobulin (1:200) for 30 minutes. Visualization was achieved by streptavidin-biotin complex conjugated with horseradish peroxidase for 30 minutes followed by diaminobenzidine chromogen solution for 10 min.

2.3.8 Statistical Analysis

PRISM 4 was used to generate graphs and for data analysis. The t-test was used to compare the means of two groups. One-way ANOVA (with Bonferroni test) was used to compare means between three or more groups. All values are presented as mean $\pm$

standard error of the mean (SEM). PRISM 4 was also used to generate scattered and Bland-Altman plot to show the agreement between T_1 obtained by IR-SE method and T_1 -effective obtained by MP-RAGE method.

In the SI time course studies, we tracked the SI over time in animals to examine the effect of $MnCl_2$ and glucose, allowing the study of dynamic relationships and differences heterogeneity. Therefore, the collected data is longitudinal data and the temporal ordering of events is important because the values that are close in time within a subject are more alike than those that are far apart. Also, the observations on the same subject are likely to be positively correlated. Generalized estimating equations (GEEs) uses regression models for repeated measures, clustered or longitudinal data sets (Zeger and Liang, 1986). Therefore, time course MEMRI SI profiles were tested for significant differences using GEE and the Mann-Whitney test used to test groups at individual time points (Stata 10, Statacorp, Texas, USA).

3 Results

3.1 Gross anatomy of the mouse pancreas

The mouse pancreas is a thin and diffuse structure (Figure 3-1). The biggest and thickest part of the mouse pancreas is the gastrosplenic portion which makes contact with 4 organs: the spleen, stomach, liver and left kidney. It is situated medial to the spleen, ventral to the upper pole of the left kidney, and inferior to the stomach and liver. The central portion of the pancreas lies adjacent to the duodenal loop, begins at the duodenal bulb and ends at the duodenal ligament. The duodenal portion of the pancreas extends into the duodenal ligament. This is the thinnest part of the mouse pancreas and is adjacent to the jejunum and colon. Therefore, the ideal MRI experiment should allow good delineation of the tissue margin.

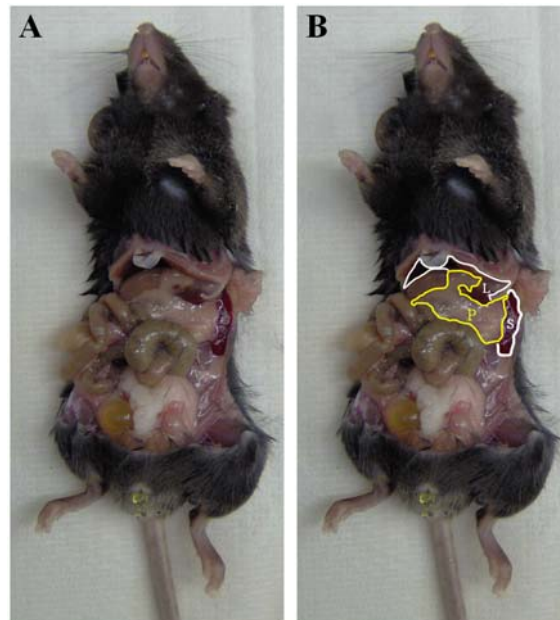


Figure 3-1. (A) Anatomic location of the pancreas within the mouse abdomen. (B) Solid lines outline margins of the pancreas (P), liver (L) and spleen (S).

3.2 Optimization of MRI

3.2.1 Spin echo

3.2.1.1 The mouse cadaver imaging

Optimization of T_1 -weighted SE images was first performed in the mouse cadaver to avoid the complication of motion artefacts. Figure 3-2 shows the SI differences between the pancreas and its surrounding organs, the kidney, liver and spleen, where various values of TR were used and TE values set at 15, 20 and 30 ms. The SI difference between the pancreas and spleen as well as between the pancreas and liver was high enough for image interpretation throughout the whole range of TR and TE values employed. The difference in the SI between tissues increased with increasing TR. In general, the SI difference between the pancreas and kidney was the lowest of the organs near the pancreas. The greatest SI difference was obtained at TR = 900 ms and TE = 15 ms and thus, these values were chosen as the optimal parameters to delineate the pancreas.

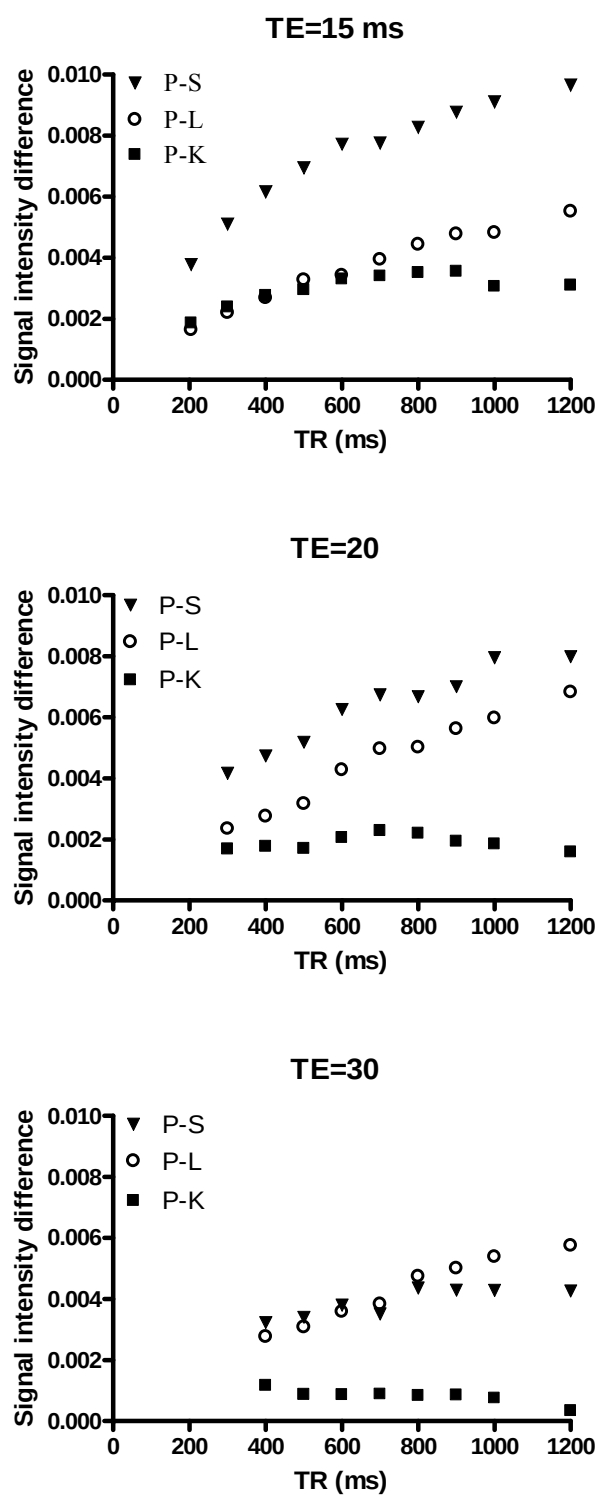


Figure 3-2. Signal intensity differences between pancreas and spleen (▼ P-S), pancreas and liver (○ P-L), and pancreas and kidney (■ P-K) at TR = 200-1200 ms, TE = 15 ms, 20 ms and 30 ms. (n = 1)

Figure 3-3A shows the optimal SE image of a mouse cadaver, image parameters were as follows: TR = 900 ms, TE = 15 ms, matrix = 256×128 , FOV = 100×50 mm, 2 averages, slice thickness = 1 mm and gap = 0.5 mm. In the mouse cadaver, the gastrosplenic portion of the pancreas can be well-delineated with reference to a standard mouse atlas (Iwaki and Hayakawa, 2005) as can other organs in the mouse abdomen (Figure 3-3B). However, the boundaries of the biliary and duodenal portions of the pancreas were indistinct.

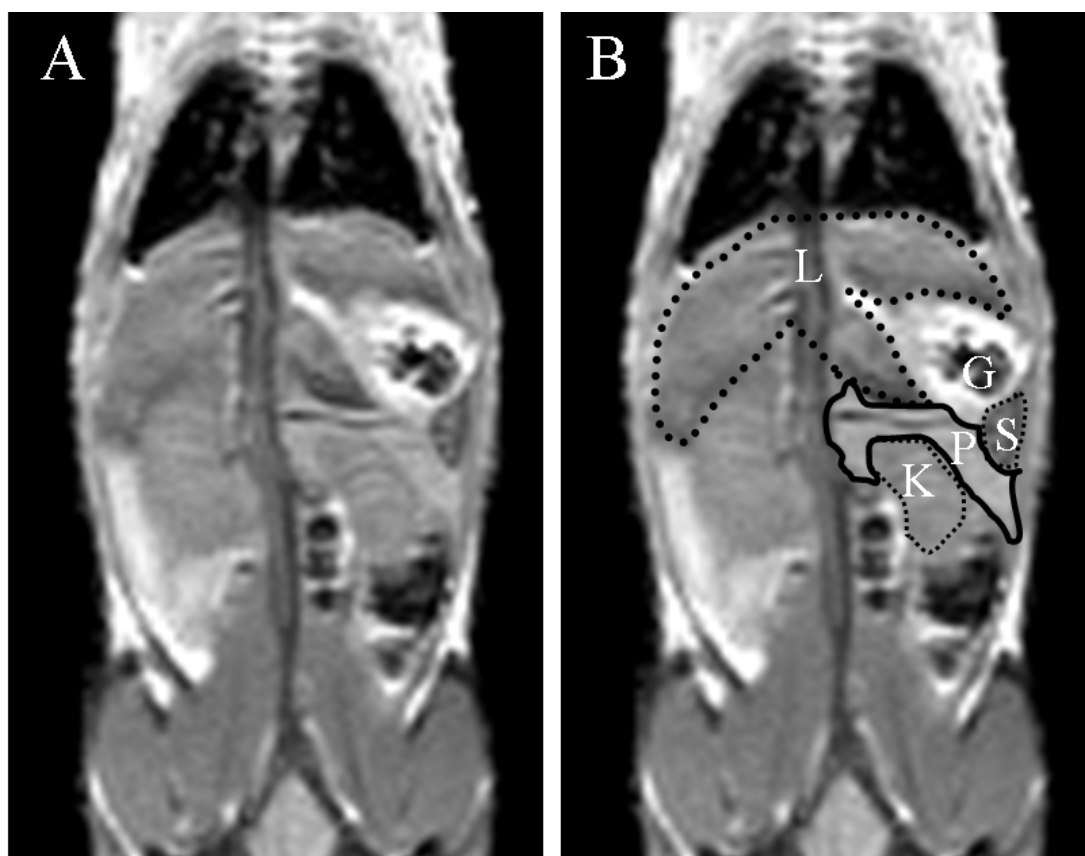


Figure 3-3. Coronal spin-echo MRI images (TR/TE = 900/15 ms) of a mouse cadaver without (A) and with (B) outline of organ margins. G: stomach, K: kidney, L: liver, P: pancreas, S: spleen.

3.2.1.2 Anaesthetized mice imaging

The optimized parameters for imaging the dead mouse pancreas (TR/TE = 900/15 ms) obtained in section 3.2.1.1 were applied to live anaesthetized mice imaging. Figure 3-4A shows the SE image collected without respiratory gating. On this image, the boundaries between solid organs and hollow organs in the mouse abdomen are indistinct due to motion artefacts caused by respiration, bowel peristalsis and the beating heart. Respiratory gating was introduced into the SE sequence such that data was collecting during expiration when respiratory motion was minimal. The depth of anaesthesia was adjusted to maintain a fixed respiratory rate of 67 beats/min to achieve TR of 900 ms. Despite considerable efforts, the respiratory rate could not be controlled well enough throughout the acquisition with the respiratory rate varying between 60-83 beats/min. Thus, tissue contrast between the pancreas and surrounding organs was insufficient to fully discern the pancreas (Figure 3-4B). However, motion artefacts were much less evident with respiratory gating and the margins of the liver, right kidney and paraspinal muscles could be readily identified (Figure 3-4B).

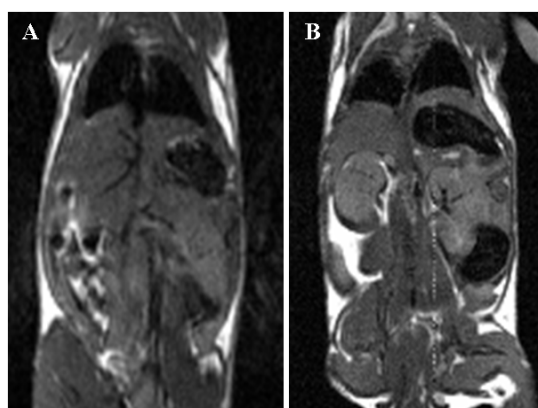


Figure 3-4. Coronal spin-echo MRI images of a live anaesthetized mouse without (A) and with (B) respiratory gating.

3.2.2 MP-RAGE

Live anaesthetized mice were scanned using a MP-RAGE sequence, considered to be relatively less susceptible to motion artefacts (section 1.6.3.4). There was less motion blur in MP-RAGE images compared to SE images even without respiratory gating, (comparing Figure 3-5A and Figure 3-4A, respectively). Further, MP-RAGE images were found to have better tissue contrast than SEMS images (comparing Figure 3-5A and Figure 3-4B, respectively). The quality of the MP-RAGE images were good enough to allow delineation of the mouse pancreas from surrounding organs (Figure 3-5B). Having established the MP-RAGE sequence was the better sequence for MRI of the mouse pancreas, parameters such as TI, flip angle and TD needed to be optimized prior to its use in subsequent MEMRI experiments.

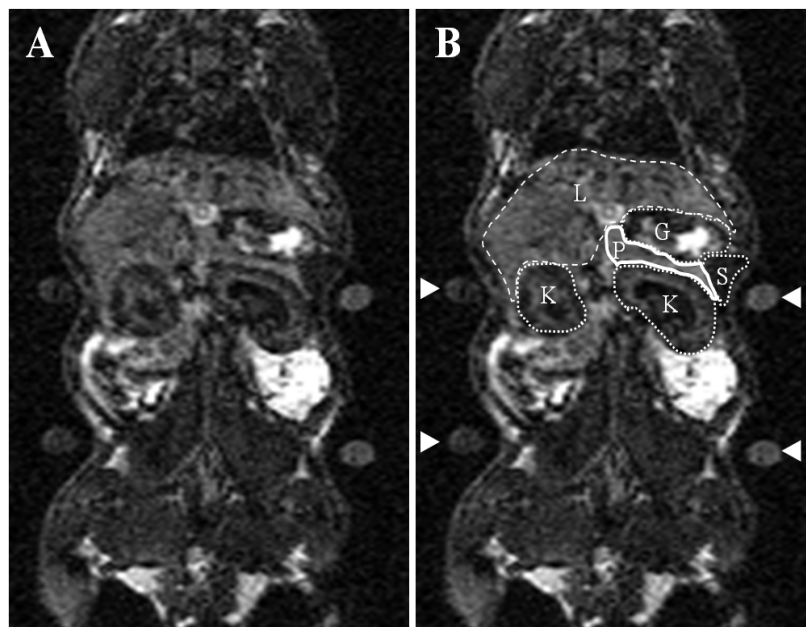


Figure 3-5. Coronal MP-RAGE MRI images of a live anaesthetized mouse without (A) and with (B) outline showing the organ margin. G: stomach, K: kidney, L: liver, P: pancreas and S: spleen. Arrowheads indicate phantoms.

3.2.2.1 TI

To achieve a positive correlation between difference in SI and accumulation of contrast agent in the tissue, the TI needs to be greater than TI_{null} . Therefore, the greatest TI_{null} for the tissues of interest was used as the preferred TI. To determine the TI_{null} , polarity-restored SI data at 12 different TIs in various tissues were fitted to signal recovery curve using equation 1: $SI(TI) = A - B \times e^{-TI/T_1}$. TI_{null} for the pancreas, muscle, spleen, kidney, liver, heart and epididymal fat were 500, 740, 680, 645, 600, 670 and 90 ms, respectively (Figure 3-6, $n = 2$). Thus, the greatest TI_{null} for these tissues was 740 ms and therefore a TI of 740 ms was chosen for subsequent experiments.

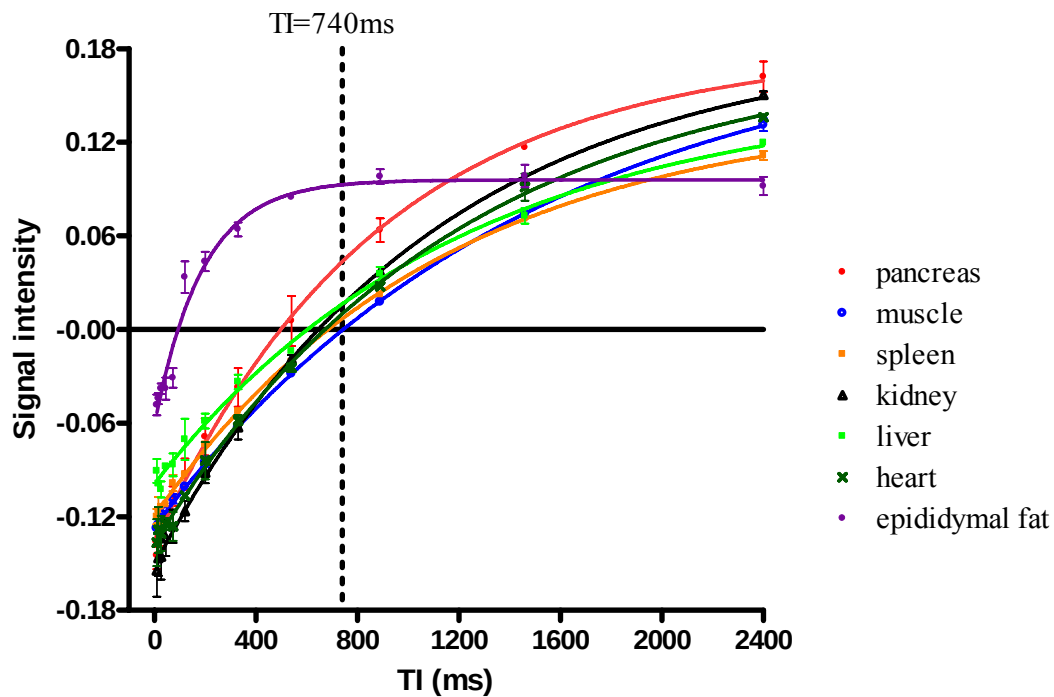


Figure 3-6. Signal recovery curves of various tissues obtained by MP-RAGE MRI ($n = 2$). Skeletal muscle has the longest TI_{null} value with a null point around $TI = 740$ ms. The dotted line indicates $TI = 740$ ms.

3.2.2.2 Flip angle

The optimal flip angle provides greatest contrast between the pancreas and surrounding tissues as well as maintaining good image quality. Therefore, differences in SI between the pancreas and adjacent tissues at various flip angles with and without MnCl_2 administration were obtained and used to determine a flip angle for subsequent MP-RAGE experiments. MP-RAGE images with a range of flip angles (5 - 50°) were performed in the same C57BL/6 mouse ($n = 1$) prior to and at 40-90 min after the start of MnCl_2 infusion (20 mM, 0.2 mL/h, 0.1 mL, taking 30 min). The MP-RAGE images with MnCl_2 infusion were acquired during the steady state signal enhancement period, 32-96 min after the start of MnCl_2 infusion (Figure 3-16). Images with diagnostic quality were obtained within the range of flip angles = 5 - 45° . A flip angle of 50° produced images with significant blurring (Figure 3-7, signal level oscillations) and so the data was not analysed and higher flip angles were not used.

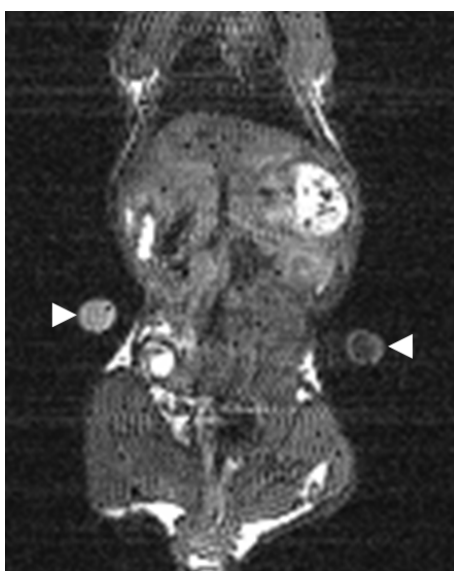


Figure 3-7. Coronal MP-RAGE MRI images of an anesthetized mouse using a flip angle of 50° . Arrowheads indicate phantoms.

Figure 3-8 shows the SI data of different organs at flip angles of 5-45° obtained from MP-RAGE MRI of C57BL/6 mouse with and without MnCl₂ infusion. In general, a similar trend in flip angle-dependent SI changes was observed for each tissue without and with MnCl₂ infusion (Figure 3-8). MnCl₂ infusion did not greatly affect the SI at different angles in the epididymal fat, interscapular fat and skeletal muscle (Figure 3-8).

A loss of SI due to low flip angle was noted in all the organs whereas a loss of SI due to high flip angle was noted in the pancreas, muscle and heart (Figure 3-8). The magnitude of the measured SI increased over the flip angle range of 5-25° and then gradually decreased as flip angles decreased from 25 to 45° in the pancreas, muscle and heart (Figure 3-8). In the spleen, liver, kidney and interscapular fat, SI increased with increasing flip angle from 5° to 20°, reaching a plateau at flip angles of 20-45° (Figure 3-8). SI increased with increasing flip angle at flip angle of 5-45° in the epididymal fat (Figure 3-8). In summary, SI increased with increasing flip angle until a flip angle of 20-25° is reached. Then, SI plateaued or decreased gradually in all tissues except the epididymal fat.

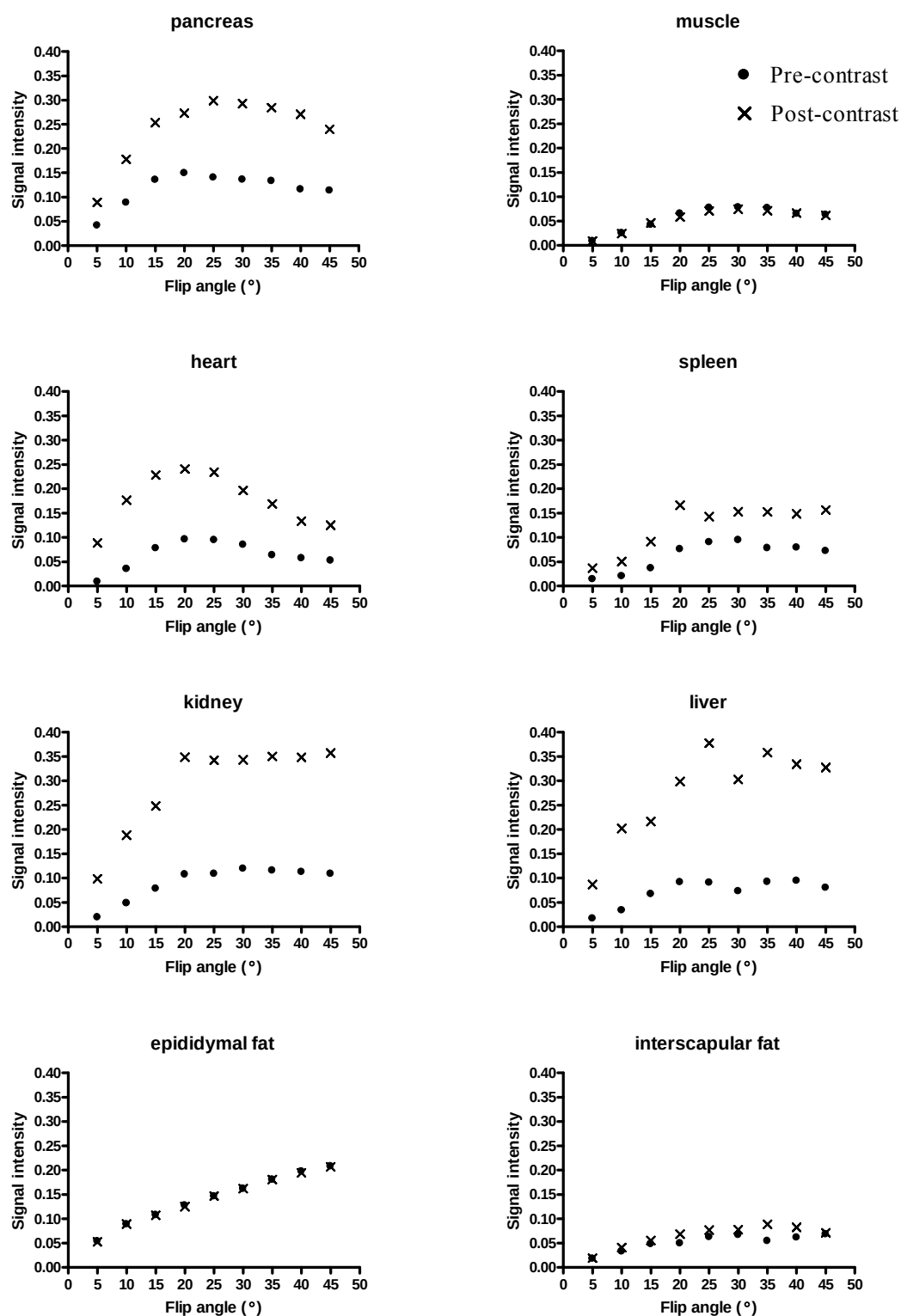


Figure 3-8. Signal intensity changes at varying flip angles of different organs of a C57BL/6 mouse with and without MnCl_2 infusion ($n = 1$).

Given that the main objective was to explore the use of MP-RAGE MRI to image β -cell activation and thus, the percentage increase in SI following MnCl_2 administration in the pancreas must be taken into account. The percentage signal enhancement in the pancreas was 83-133 % over the flip angle range 2-45° after a MnCl_2 dose (20 mM, 0.1 mL, 0.2 mL/h) for 30 min (Figure 3-9, $n = 1$). The whole range flip angles provided a detectable change in SI in the pancreas following MnCl_2 infusion.

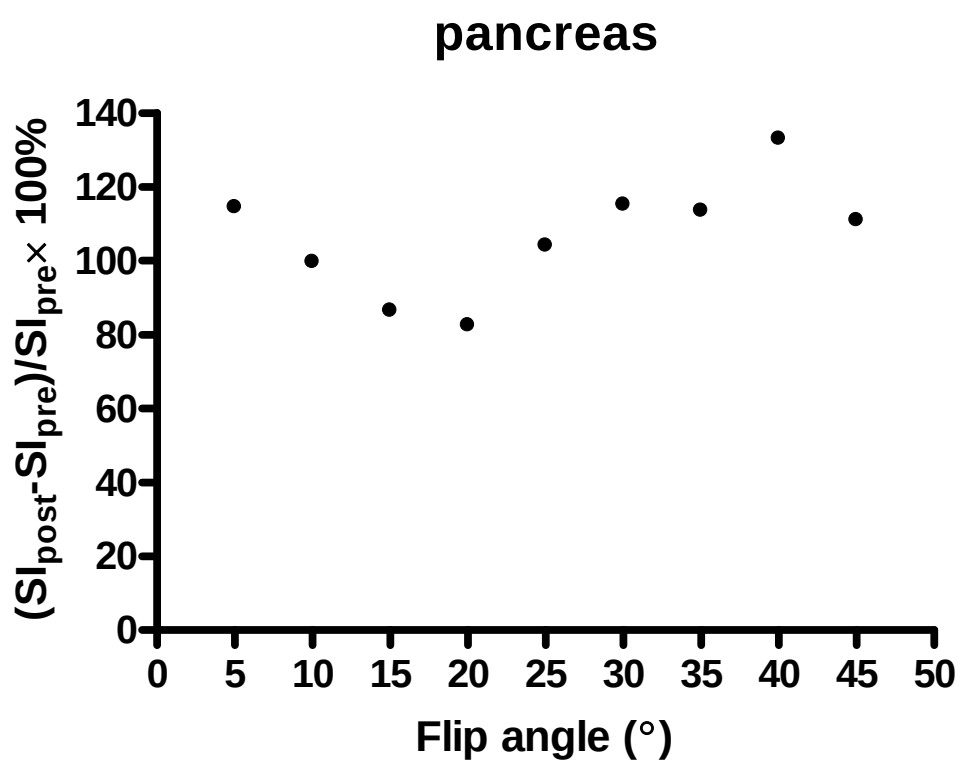


Figure 3-9. Percentage change of signal intensity in the pancreas pre- and post- MnCl_2 infusion (20 mM, 0.1 mL, 0.2 mL/h) in an anesthetized mouse ($n = 1$).

While SI of the various organs are important, the tissue contrast between the pancreas and its adjacent organs is also crucial for MRI of the pancreas. Figure 3-10A shows the SI difference between the pancreas and its surrounding organs including the kidney, liver and spleen in MP-RAGE MRI images without MnCl_2 administration. In general, SI differences increased over the flip angle range $5\text{-}15^\circ$, peaked at 15° and then decreased for values of $15\text{-}45^\circ$. Tissue contrast was highest between the pancreas and spleen, and lowest between the pancreas and kidney. The SI difference between the pancreas and spleen, and between pancreas and liver, was high enough throughout the range of flip angles for delineation of the pancreas from both the spleen and liver.

After MnCl_2 administration (Figure 3-10B), there was no clear trend of the SI difference between the pancreas and surrounding organs. However, the SI difference between the pancreas and surrounding organs was enough throughout the range of flip angles for delineation of the pancreas from the surrounding organs except at a flip angle 5° at which there was no difference in the SI between the pancreas and liver. Therefore, a flip angle of 5° was not recommended according to Figure 3-10.

MP-RAGE MRI is acquired with low flip angles. However, the acquired SI was low by using low flip angle (Figure 3-8). Figure 3-10 showed a flip angle of 10° was the smallest one which provided readily delineation of the pancreas from surrounding organs and also achieved adequate signal enhancement in the pancreas following MnCl_2 infusion. Therefore, a flip angle of 10° was recommended as a compromise between delineation of the pancreas and signal enhancement following MnCl_2 administration.

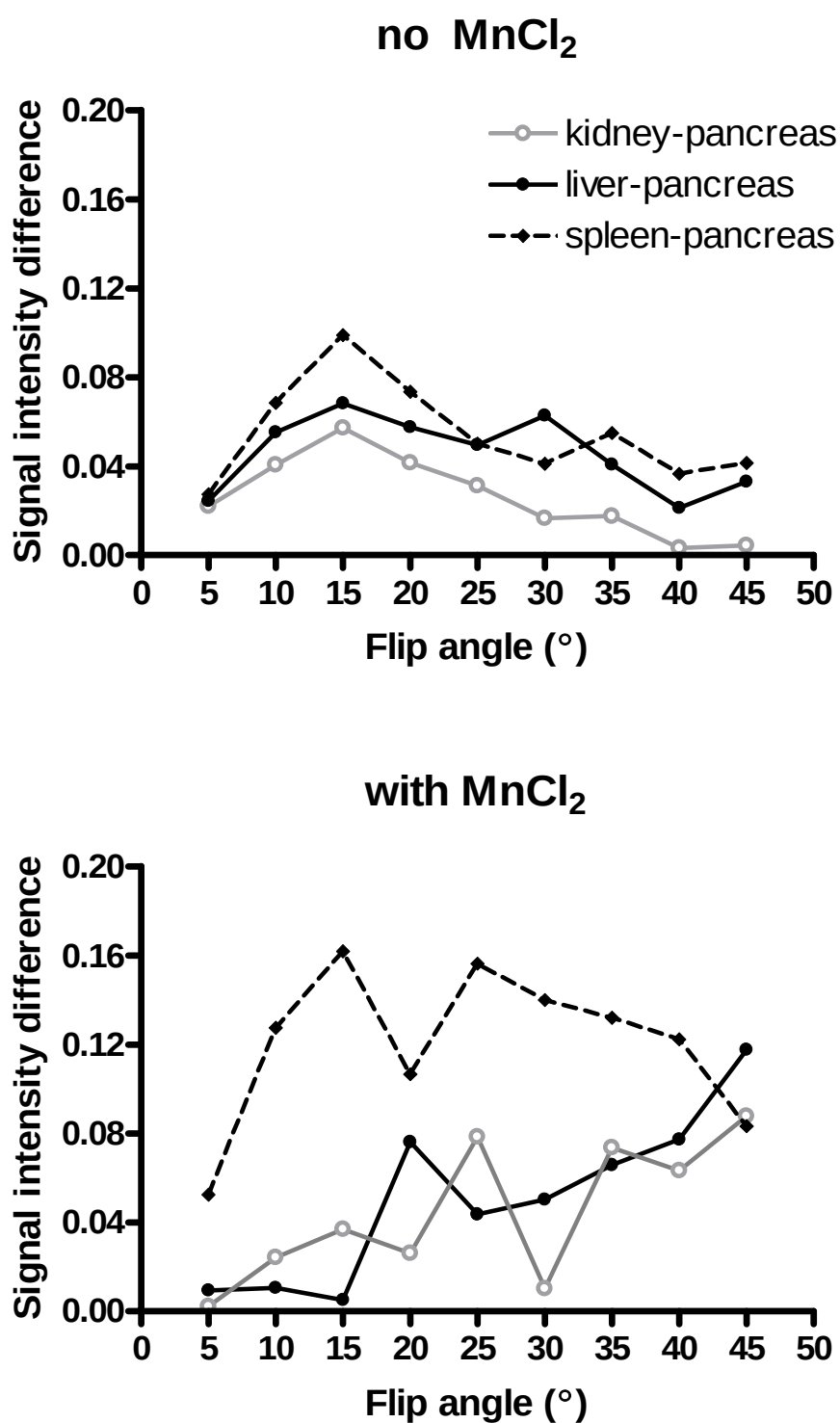


Figure 3-10. Signal intensity differences between pancreas and its surrounding organs, the kidney, liver and spleen, at different flip angles in an anesthetized C57BL/6 mouse (n = 1) without and with MnCl_2 enhancement.

3.2.2.2.1 Delay time (TD)

The scan time for a MP-RAGE sequence is dependant on $TR_0 \times \text{phase encoding steps in outer loop}$, where $TR_0 = TI + (TR \times \text{phase encoding steps in inner loop}) + TD$. Clearly, TI, TD and matrix size affects scan time in MP-RAGE sequence. Ideally, TR_0 should be set to greater than $5 \times T_1$ of longest T_1 organ to achieve 99 % recovery of longitudinal magnetization

Previously, T_1 in the pancreas, kidney, liver, muscle and fat of mouse cadavers have been measured to be $\sim 550, 800, 625, 825$ and 400 ms, respectively by using IR-SE in our group by Sahuri and Herlihy (personal communication 2007). In this case, the highest T_1 is ~ 825 ms in skeletal muscle. Thus, TR_0 should be $825 \text{ ms} \times 5 = 4125$ ms. With imaging parameters of $TI = 740$ ms, $TD = 2000$ ms and matrix size = $256 \times 128 \times 64$, the resultant TR_0 in MP-RAGE sequence is 3380 ms which is $> 6 \times T_1$ for the pancreas and $> 4 \times T_1$ for skeletal muscle (linear ordering). Thus, TD was set to be 2000 ms as a compromise between shortening scan time and fully longitudinal magnetization relaxation.

3.2.2.3 Summary

The optimized MP-RAGE MRI parameters were as follows: $TI = 740$ ms, flip angle = 10° , $TD = 2000$ ms, $FOV = 100 \times 50 \times 32$ mm (or $80 \times 40 \times 20$ mm), matrix = $256 \times 128 \times 64$ mm and 1 scan. With these parameters, the majority of the mouse pancreas can be delineated from the surrounding tissues with $MnCl_2$ infusion (Figure 3-11). Employment of respiratory and cardiac gating provides images with less motion

artefacts. However, it results in a range of TI in the MP-RAGE image. Since MP-RAGE MRI was shown to provide diagnostic-quality images, respiratory and cardiac gating was not employed to the sequence.

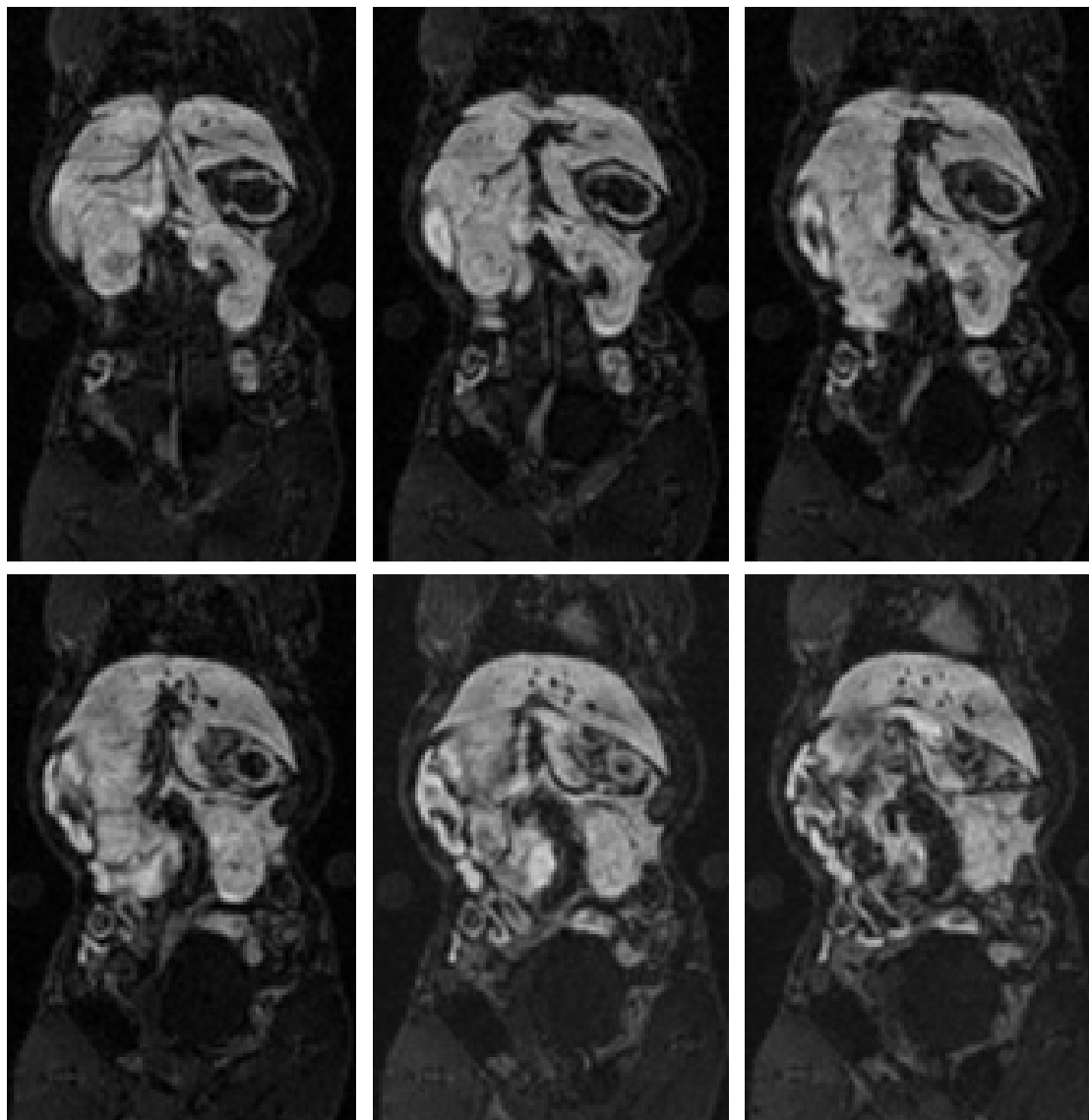


Figure 3-11. Consecutive coronal MP-RAGE MRI images showing the pancreas of a C57BL/5 mouse at 40-90 min after the start of MnCl_2 infusion *in vivo*. Note: Solid lines demonstrate the margin of the mouse pancreas

3.3 Validation of MP-RAGE T_1 measurement

IR-SE is the accepted standard for determining T_1 values. However, as discussed previously, SE-based images failed to delineate the mouse pancreas from the surrounding tissues. Therefore, the possibility of using MP-RAGE MRI for T_1 measurement was explored. In this study, an *in vitro* experiment was performed to determine the application of the MP-RAGE method for measurement of T_1 . The acquired MP-RAGE data were fitted to the equation 1: $SI(TI) = A - B \times e^{-TI/T_1}$ for T_1 estimation and the resultant T_1 values were defined as $T_{1\text{-effective}}$ to distinguish the T_1 measurement from that obtained by IR-SE.

Three T_1 measurement methods were performed in samples containing $MnCl_2$ solutions at the concentrations of 0.04-1.00 mM: (1) IR-SE method with 18 data points and $TR = 15$ s, (2) MP-RAGE method with 18 data points and $TD = 15$ s and (3) MP-RAGE method with 5 data points and $TD = 2$ s. The first two methods used a long TR or TD up to 15 s to achieve full recovery of longitudinal magnetization ($> 5 \times T_1$ of all samples). The third method used a shorter TD and therefore achieved lesser degree of longitudinal magnetization recovery.

The IR-SE and MP-RAGE data acquired with different TI values were found to be well-described by the IR equation (equation 1, see above) with a goodness of fit (R^2) > 0.99 at $MnCl_2$ concentrations of 0.04-1.00 mM even by the MP-RAGE method with a shorter TD of 2 s (Figure 3-12). Therefore, the IR equation may be applied to fit the MP-RAGE MRI data at various TIs for obtaining T_1 values.

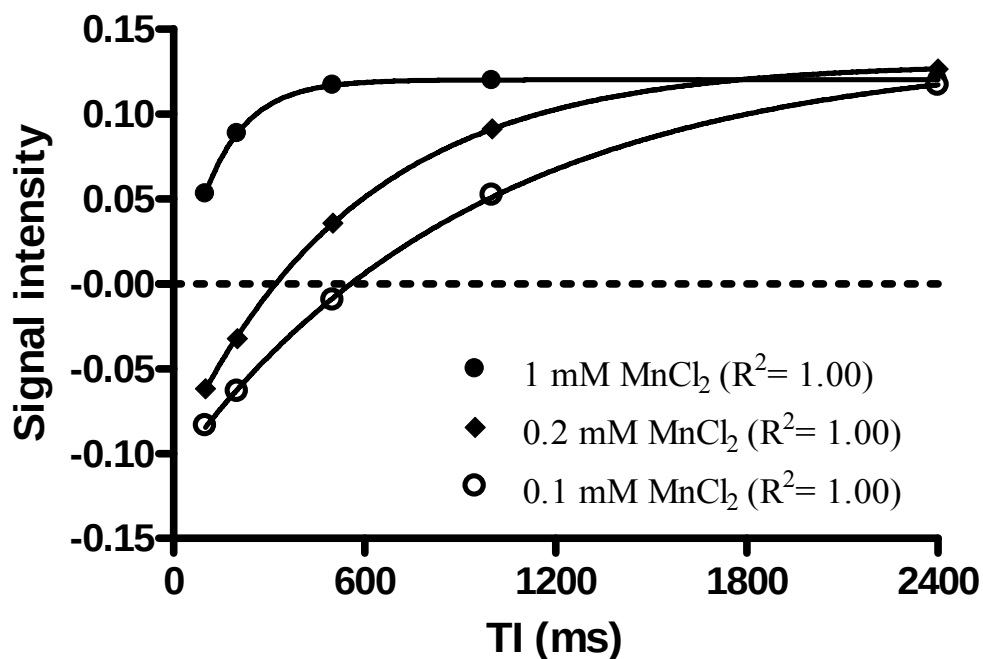


Figure 3-12. Polarity-restored signal recovery curves obtained for 3 different concentrations of MnCl_2 solutions by MP-RAGE method with $\text{TD} = 2$ s.

By these three T_1 measurement methods, increasing $[\text{Mn}]$ led to a decrease in T_1 and T_1 -effective (Figure 3-13), consistent with the use of MnCl_2 as a MRI contrast agent. In addition, there exists a good positive correlation between the R_1 or R_1 -effective values and the MnCl_2 concentrations by these three methods ($R^2 > 0.99$, Figure 3-13). Therefore, these three methods can be used for a quantitative estimation of changes in $[\text{Mn}^{2+}]$ *in vitro*.

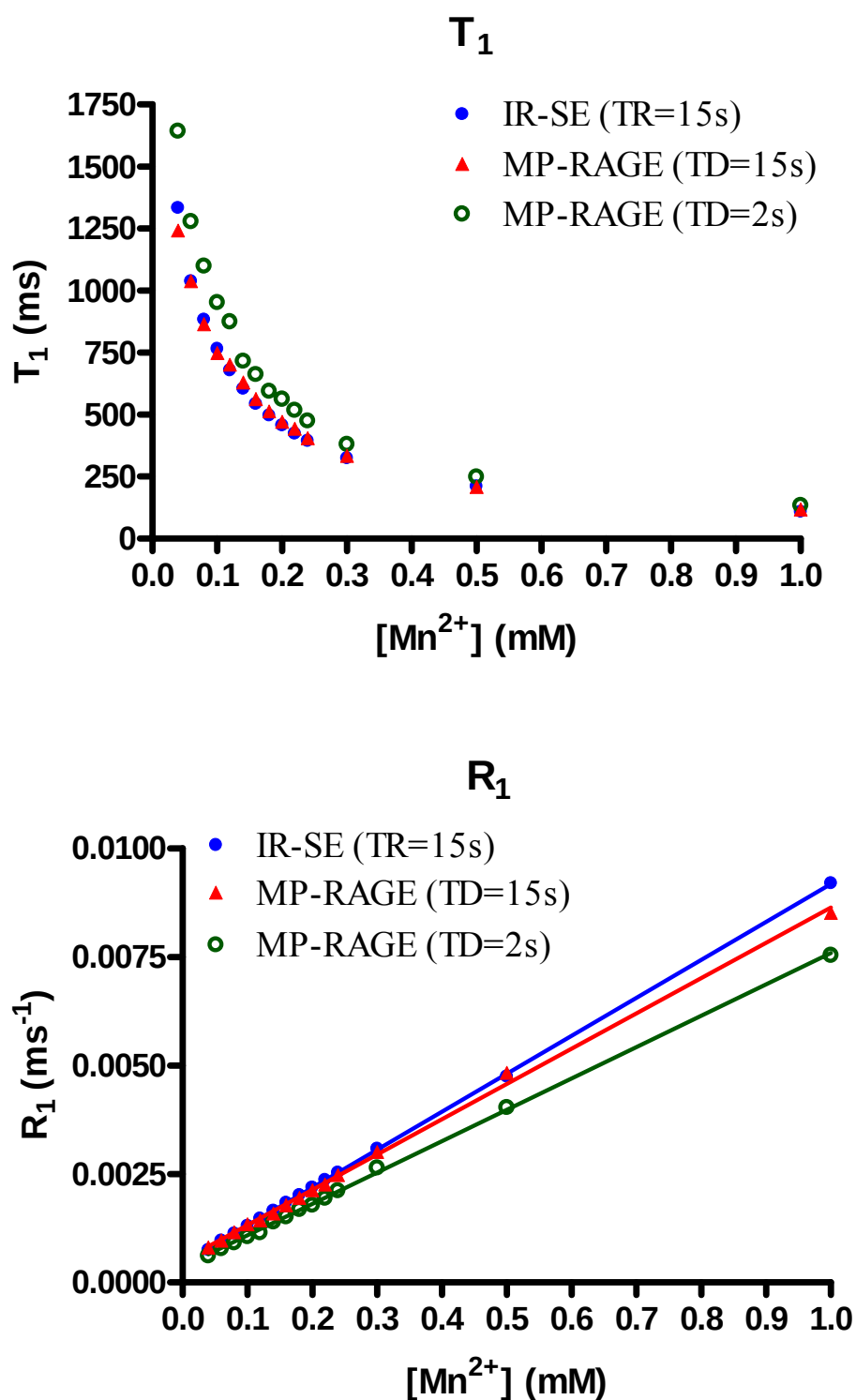


Figure 3-13. T_1/T_1 -effective and R_1/R_1 -effective values obtained by IR-SE method with TR = 15 s, MP-RAGE method with TD = 15 s and MP-RAGE method with TD = 2 s for increasing concentrations of $MnCl_2$ solutions.

There is a strong positive correlation between the T_1 values by IR-SE method with TR = 15 s and T_1 -effective values obtained by MP-RAGE method with TD = 15 s for different concentration of $MnCl_2$ solution (Figure 3-14A, $R^2 > 0.99$). The mean difference of T_1/T_1 -effective values obtained by these two methods is 1.5 % and the limits of agreement are from -5.8 to 8.8 % (Figure 3-14B). Thus, there is a good agreement in the IR-SE and MP-RAGE T_1 measurement methods, when the full recovery of longitudinal magnetization is achieved.

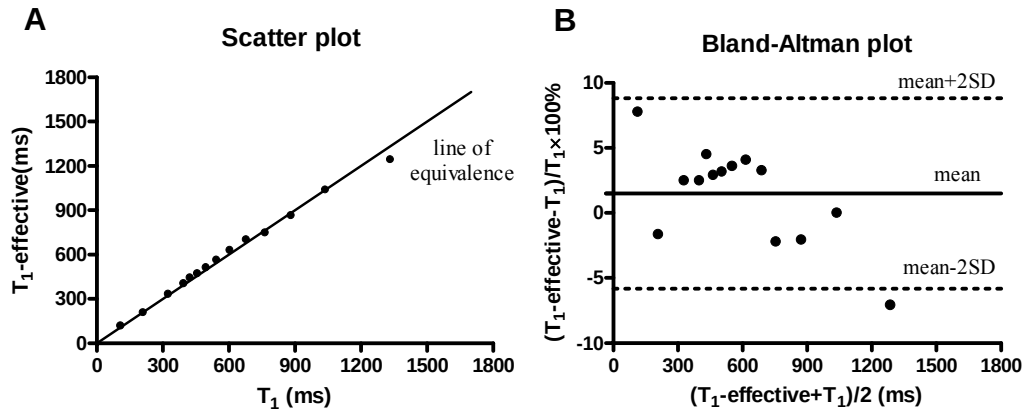


Figure 3-14. Scatter plot (A) and Bland-Altman plot (B) of T_1 and T_1 -effective values obtained by IR-SE method and MP-RAGE (TD = 15 s), respectively. Solid line in Bland-Altman plot represents the mean difference between measurements, and dashed lines represent 2 standard deviations above and below the mean difference.

Figure 3-15A compares T_1 values obtained by IR-SE method (TR = 15 s), and T_1 -effective values obtained by MP-RAGE method with TD = 2 s, for different concentrations of $MnCl_2$, showing T_1 -effective values are higher than the corresponding T_1 values. Thus, T_1 -effective values obtained by MP-RAGE method do

deviate from the true T_1 as measured by standard IR-SE method) but T_1 and T_1 -effective values are highly correlated ($R^2 > 0.99$, Figure 3-15A). With the Bland-Altman plot, the level of agreement between the two methods, IR-SE and MP-RAGE, of measuring T_1 values was assessed (see Figure 3-15B). The mean difference of T_1/T_1 -effective values obtained by these two methods is 19.6 % and the limits of agreement are from 14.7 to 24.6 % (Figure 3-15B), indicating that the T_1 -effective values obtained by MP-RAGE tended to overestimate T_1 values.

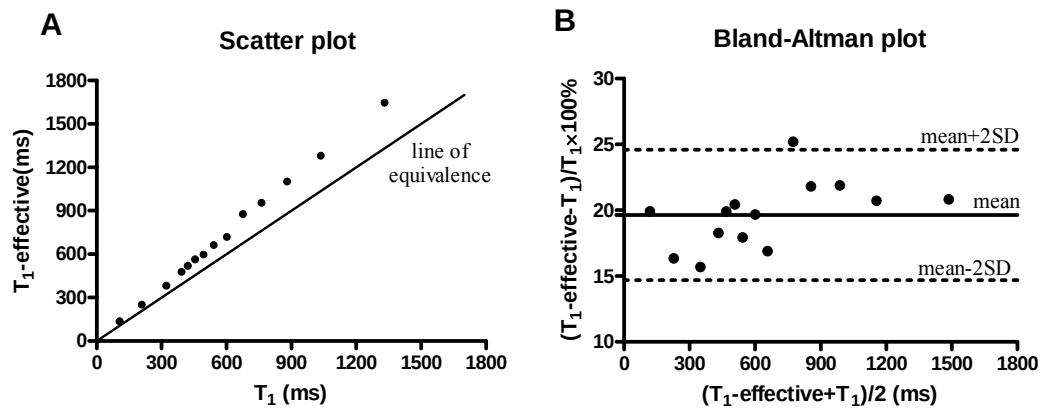


Figure 3-15. Scatter plot (A) and Bland-Altman plot (B) of T_1 and T_1 -effective values obtained by IR-SE method and MP-RAGE (TD = 2 s), respectively. Solid line in Bland-Altman plot represents the mean difference between measurements, and dashed lines represent 2 standard deviations above and below the mean difference.

Conventionally, tissue T_1 values are measured using IR-SE. In this study, the MP-RAGE methods for T_1 measurement (TD = 2 and 15 s) led to an overestimation of T_1 . There was a better agreement between the MP-RAGE T_1 measurement method with TD = 15 and the IR-SE method (Figure 3-14B vs. Figure 3-15B). However, the scan

time should always take into consideration in *in vivo* MRI study. Therefore, MP-RAGE T_1 measurement method with $TD = 2$ s was used for *in vivo* study as a compromise between accurate T_1 value and scan time.

3.4 Dose-dependent effects of MnCl_2

3.4.1 Serial MEMRI

Tissue SI changes following administration of different doses of MnCl_2 (2, 5, 10, 20 and 40 mM; 0.1 mL; 0.2 mL/h) is shown in Figure 3-16 ($n = 3/\text{group}$). All animals experienced an increase in respiratory rate within 4 min after all doses of MnCl_2 infusion, but respiratory rate reverted toward normal or below by the end of infusion. In general, SI rose during MnCl_2 infusion and reached a maximum at the end of MnCl_2 infusion in the pancreas, kidney and liver (Figure 3-16A-C). The maximum enhancement plateau in these organs lasted until the latest time point of the experiment (~ 60 min after the end of MnCl_2 infusion). There was a positive correlation between infused MnCl_2 dose and SI changes in these organs which uptake Mn^{2+} . Increasing the infused MnCl_2 dose had a marked effect on the overall SI, but provided different shaped dose-response curves and saturation levels. In contrast to the relatively steady state of enhancement in the plateau phase in other organs, the plateau phase in the liver had greater fluctuations (Figure 3-16C).

There was no such dose-dependent response after various MnCl_2 doses in the skeletal muscle and spleen. In skeletal muscle, there was no significant difference between SI profiles following different doses of MnCl_2 except after the administration 0.1 mL MnCl_2 at 40 mM (Figure 3-16D). After the administration of 0.1 mL MnCl_2 at 40 mM, there was an early wash-in and followed by gradual wash-out of SI in skeletal muscle (Figure 3-16D). In the spleen, there was a general increase in the SI profiles following MnCl_2 infusion (Figure 3-16E). However, the steady-state signal enhancement in the

spleen was not correlated to the infused MnCl_2 . After the administration of 0.1 mL MnCl_2 at 40 mM, the signal enhancement pattern in the spleen was similar to that in skeletal muscle.

In the heart, epididymal fat and interscapular fat, SI data could not be measured from the same ROI throughout the entire serial MEMRI due to motion artefacts and the data was not included here.

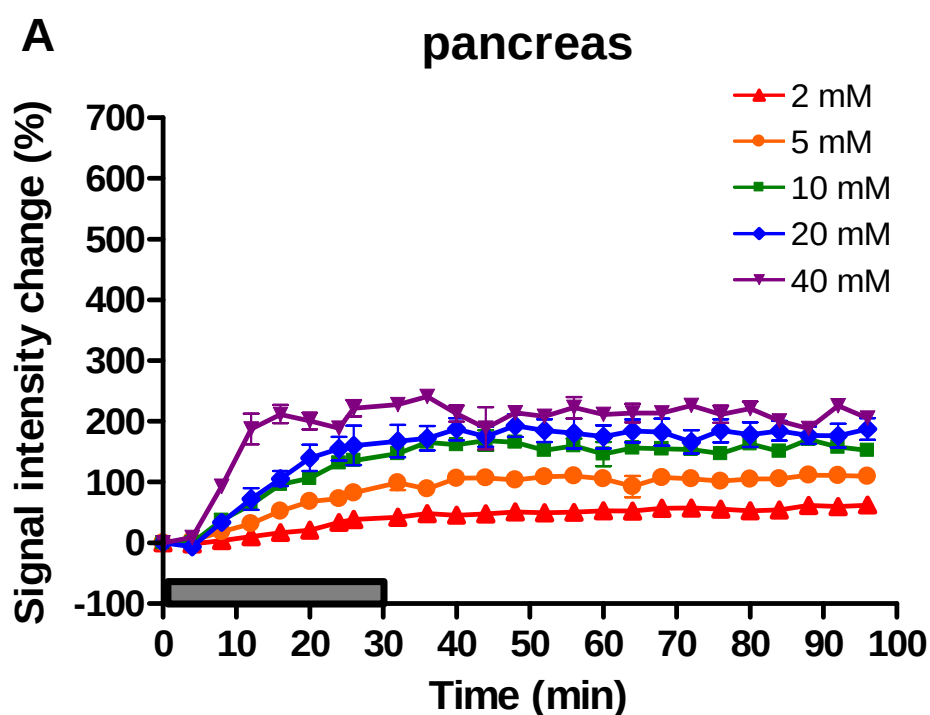


Figure 3-16. Dose-dependent signal intensity time-course in the (A) pancreas, (B) kidney, (C) liver, (D) muscle and (E) spleen following MnCl_2 infusion ($n = 3/\text{group}$). The grey bar indicates the duration of MnCl_2 infusion. Data are presented as mean $\pm$ SEM. All graphs are plotted on the same scale.

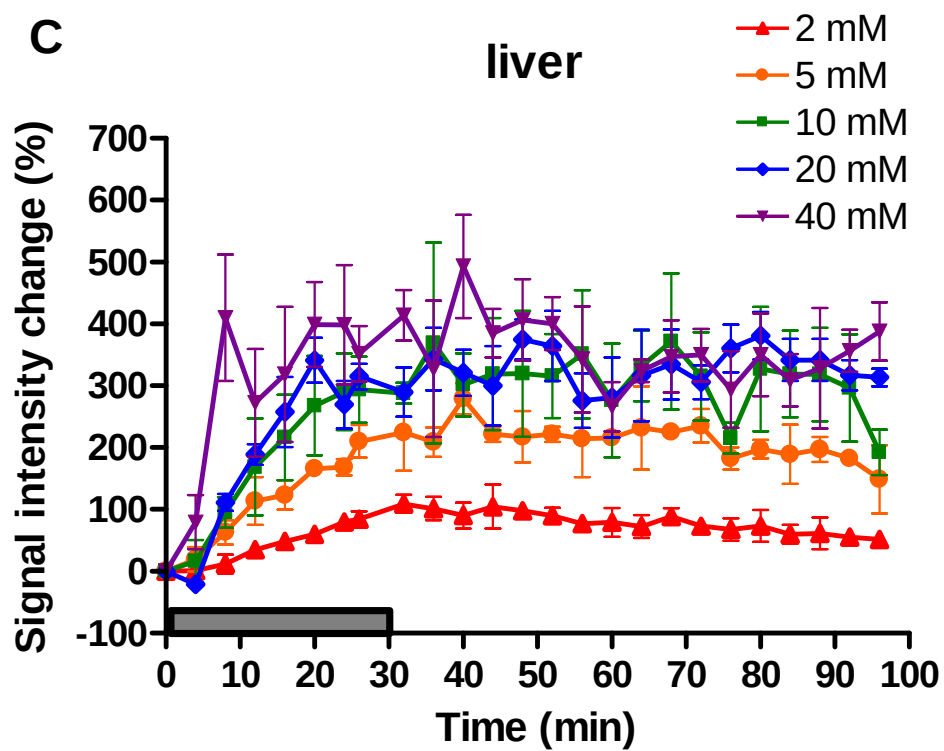
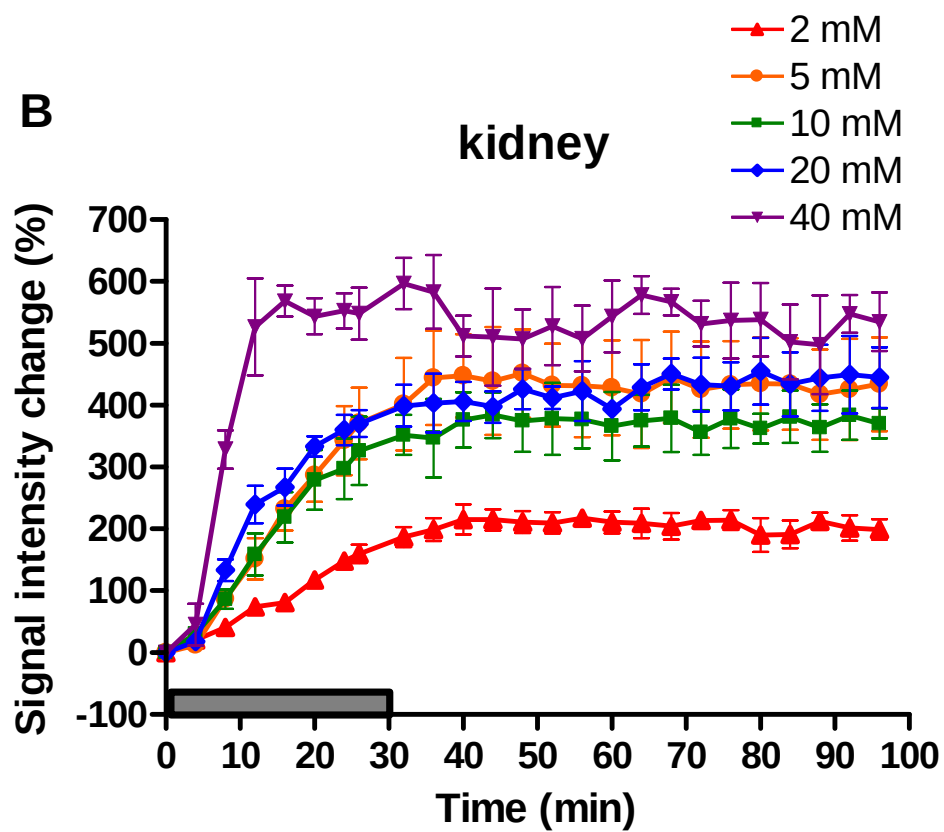


Figure 3-16. Continued.

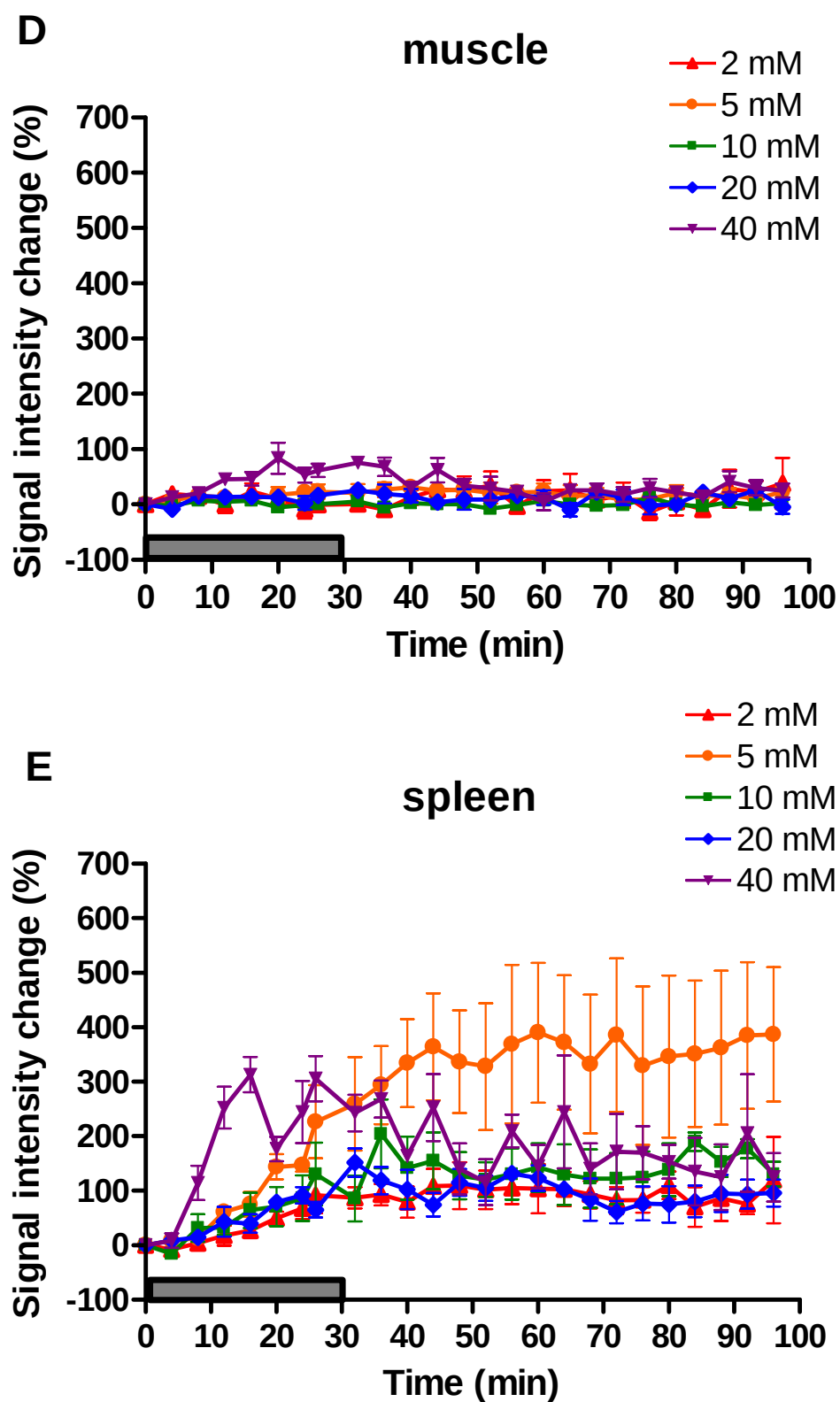


Figure 3-16. Continued.

3.4.2 T_1 measurement

Figure 3-17 shows the tissue T_1 -effective values after infusion of different doses (2, 5, 10, 20 and 40 mM; 0.1 mL; 0.2 mL/h) of $MnCl_2$ ($n = 4/\text{group}$). The control group consisted of 6 animals without contrast administration. T_1 -effective values were measured 36 min after the start of the $MnCl_2$ infusion.

T_1 -effective decreased with increasing $MnCl_2$ doses in the pancreas, liver and kidney ($p < 0.001$, one-way ANOVA, Figure 3-17). In the heart and spleen, there was a dose-dependent relationship between infused $MnCl_2$ doses and the decrease in T_1 -effective values, although this was non-linear. The highest dose of $MnCl_2$ (40 mM, 0.1 mL, 0.2 mL/h) in this study did not reach saturation in these organs. While in the epididymal fat, the T_1 -effective values remained unaffected throughout the whole $MnCl_2$ dose range ($P > 0.05$, one-way ANOVA). The T_1 -effective values in skeletal muscle also remained unaffected at $MnCl_2$ concentrations of 0-20 mM ($P > 0.05$, one-way ANOVA). The highest $MnCl_2$ dose group in skeletal muscle was not compared here as the previous result (see Figure 3-16D) shows an increase in skeletal muscle SI after the administration of 0.1 mL $MnCl_2$ at 40 mM.

In the interscapular fat, there was a significant difference in T_1 -effective values between groups with different doses of $MnCl_2$ infusion ($P < 0.05$, one-way ANOVA). However, there was not a correlation between infused $MnCl_2$ doses and change in T_1 -effective values. Therefore, MRI data of the interscapular fat data obtained by this method may be unreliable for estimating tissue [Mn].

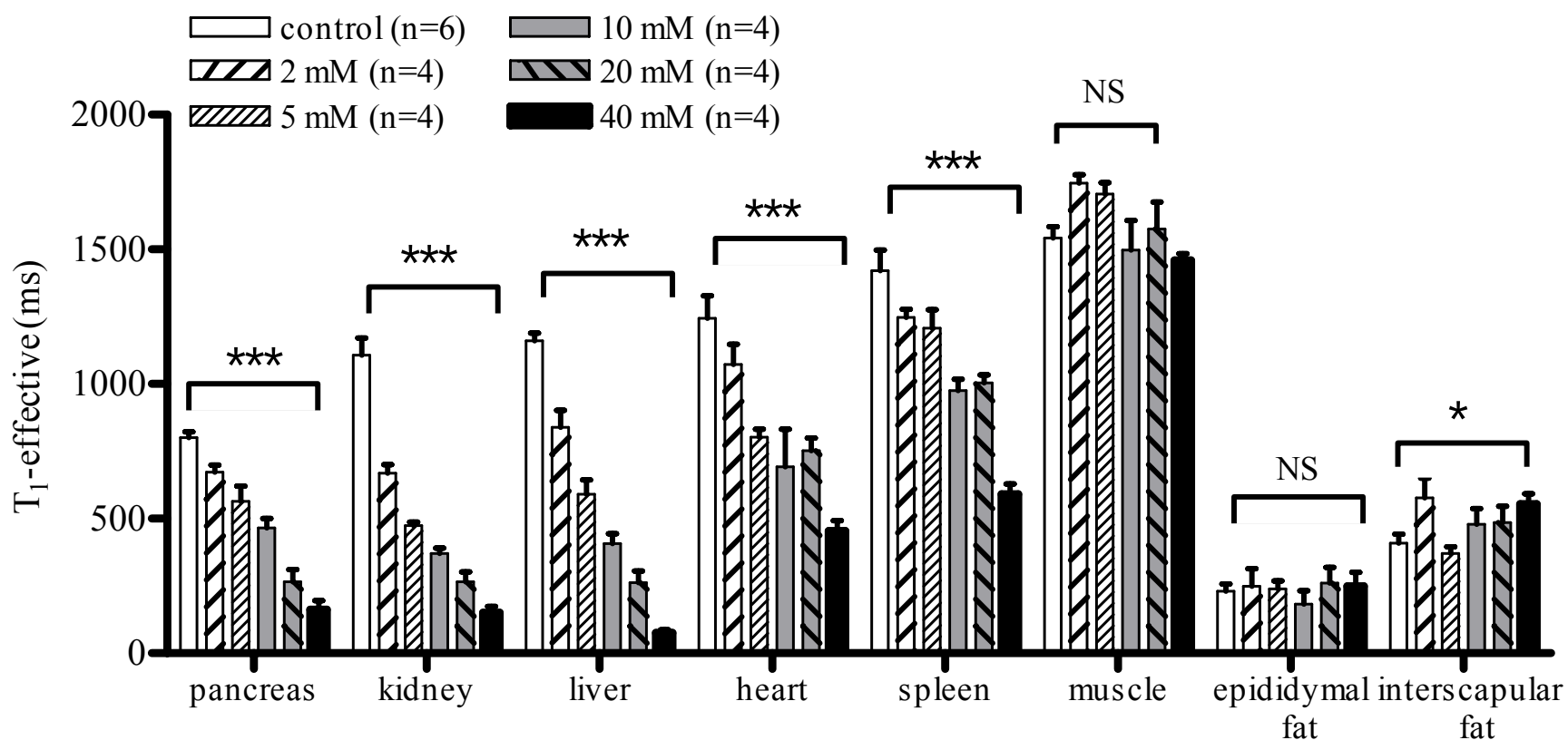


Figure 3-17. Dose-dependent T_1 -effective in mouse organs in increasing $MnCl_2$ doses (2, 5, 10, 20 and 40 mM, 0.1 mL; $n = 4$ /group). The control group were not infused ($n = 6$). ***= $p < 0.001$ by one-way ANOVA. Data are presented as mean $\pm$ SEM.

3.4.3 Tissue [Mn] analysis by ICP-AES

Tissue Mn assessed by ICP-AES showed increasing tissue [Mn] with increasing infused MnCl_2 doses ($n = 3/\text{group}$) for the kidney, pancreas, liver and heart (Figure 3-18A). The strong positive linear correlation in the pancreas ($R^2 = 0.9714$), kidney ($R^2 = 0.9775$), liver ($R^2 = 0.9237$) and heart ($R^2 = 0.9598$) indicates a dose-dependent Mn uptake. In addition, greater Mn accumulation was observed in the liver, kidney and pancreas compared to the heart (Figure 3-18A).

In the spleen, skeletal muscle and epididymal fat, infusion of up to 40 mM of MnCl_2 had no significant effect on the concentrations of Mn by ICP-AES (Figure 3-18B). Tissue [Mn] was greater in these tissues in which the animals received MnCl_2 infusion compared to that not infused with MnCl_2 , however, a dose-dependent effect was not observed (Figure 3-18B).

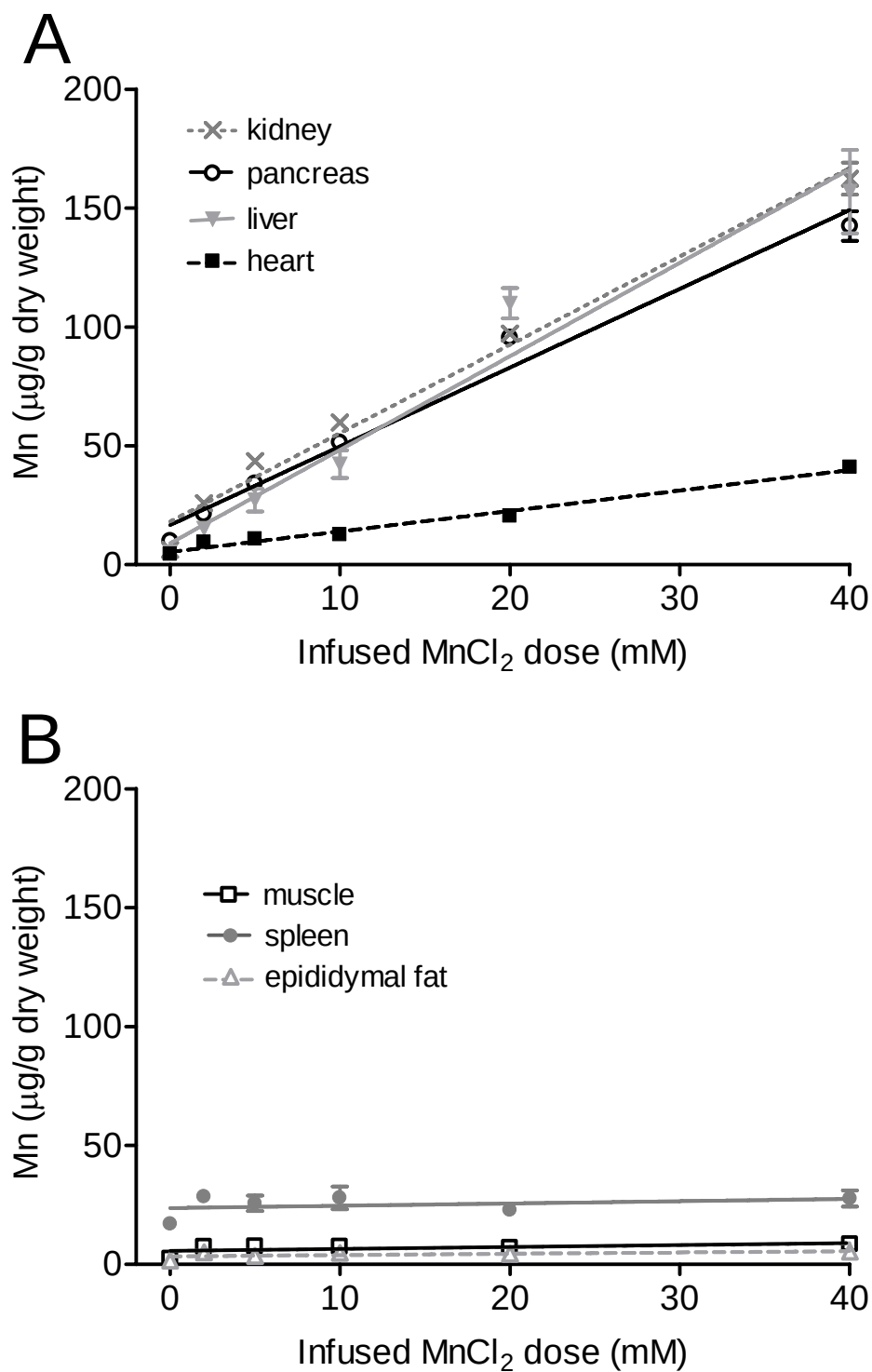


Figure 3-18. Dose-dependent changes in tissue manganese concentrations as measured by ICP-AES ($n = 3/\text{group}$). Data are presented as mean $\pm$ SEM.

3.4.4 Correlation of tissue R_1 -effective with tissue [Mn]

As [Mn] increased, the R_1 -effective values also increased in the pancreas, kidney, liver and heart (Figure 3-19). A strong linear correlation between R_1 -effective values and tissue Mn content, as measured by ICP-AES, was obtained in the pancreas ($R^2 = 0.7129$), kidney ($R^2 = 0.8563$) and heart ($R^2 = 0.8070$) (Figure 3-19). There were two different slopes for fitting the linear dose response curve in the liver. The correlation for the liver R_1 -effective values and tissue [Mn] was 0.7527 for doses ranging 2-20 mM of $MnCl_2$. At $MnCl_2$ infused doses greater than 20 mM, there appeared to be a greater effect of tissue [Mn] on the R_1 -effective. This unexpected response may be explained if the relationship between R_1 and [Mn] is non-linear. This may be the case, as in our *in vitro* study, we have only tested the R_1 -effective below 0.01 ms^{-1} (Figure 3-13B). It was not possible to determine if there was a linear response between R_1 -effective values and tissue Mn content in the spleen, skeletal muscle and epididymal fat since a wide range of [Mn] was not obtained at the different $MnCl_2$ doses.

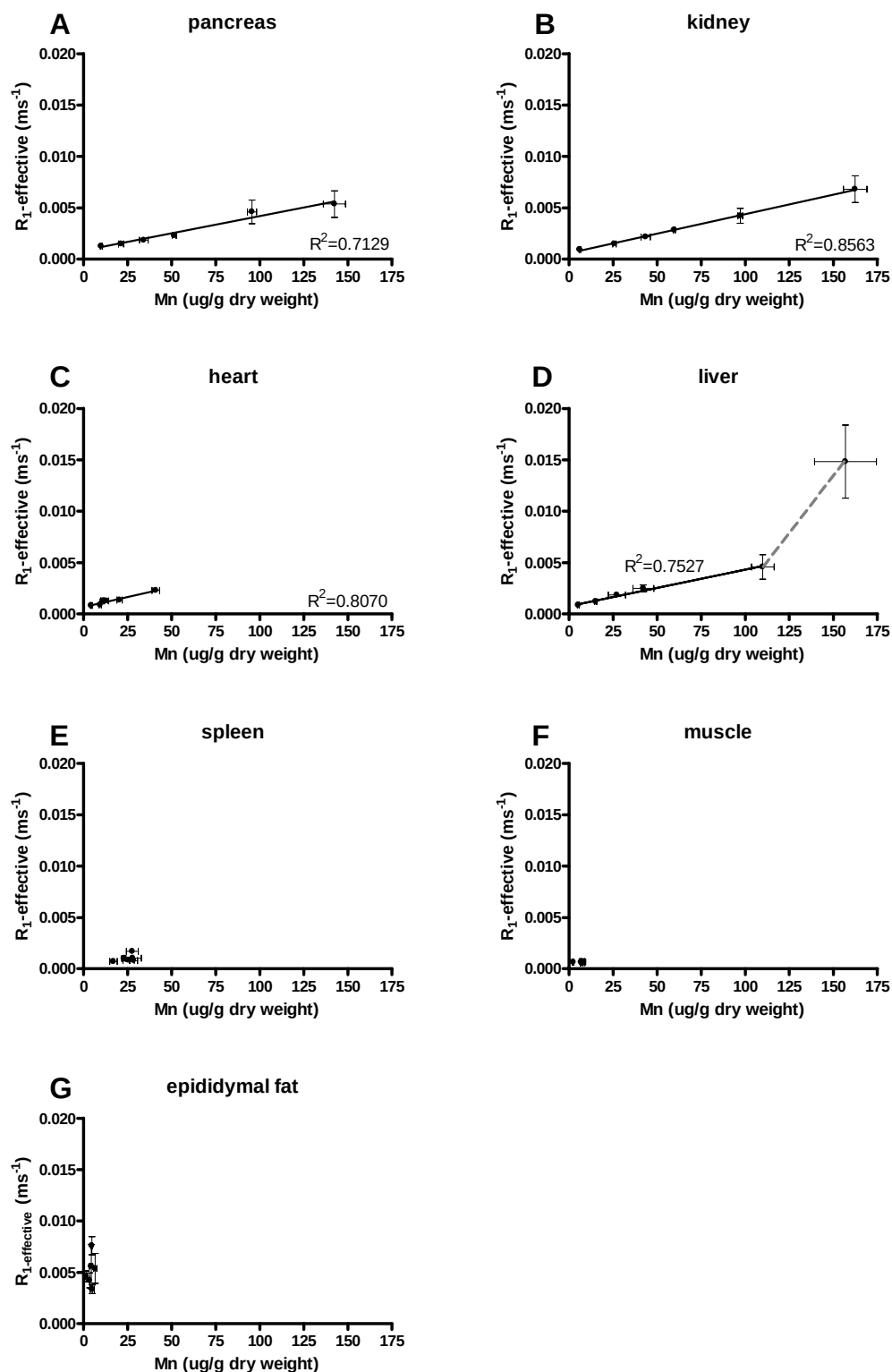


Figure 3-19. Correlation between R_1 -effective obtained by MP-RAGE and tissue manganese content by ICP-AES in the (A) pancreas, (B) kidney, (C) heart, (D) liver, (E) spleen, (F) muscle and (G) epididymal fat. Data are presented as mean $\pm$ SEM.

3.4.5 Summary

Changes in T_1 -effective values and SI were both dose-dependent and tissue-variable except in the adipose tissue and skeletal muscle. Therefore, MP-RAGE MRI can be used to obtain semi-quantitative measurements of tissue [Mn] according to the SI and T_1 measurement data. This is supported by *in vitro* tissue [Mn] measurement by ICP-AES. $MnCl_2$ dose at a concentration of 2 mM, an infusion rate of 0.2 mL/h and a total volume of 0.1 mL (about 2 mg/kg) was found to be the lower infused dose providing significant signal enhancement in the pancreas. This is the optimal $MnCl_2$ dose to minimize toxicity but still provide adequate tissue contrast for localization of mouse pancreas by MRI. Further, this dose is well below the LD_{50} of i.v. $MnCl_2$ at 38 mg/kg (Silva *et al.*, 2004), and so was used for subsequent *in vivo* studies.

3.5 Tissue manganese washout

Tissue Mn^{2+} washout in the mouse organs were assessed by longitudinal measurements of T_1 -effective values. A single dose of MnCl_2 infusion (2 mM, 0.2 mL/h, 0.1 mL) was infused and T_1 -effective measurements were performed 24 h prior to (- 24 h) and 1, 4, 24, 48 and 120 h after the start of MnCl_2 infusion. Mice were recovered from anaesthesia and had free access to standard chow between scanning. All animals ($n = 3$) survived till the end of this experiment (120 h after MnCl_2 infusion) without any obvious side effects.

In the pancreas, kidney and liver, T_1 -effective values decreased rapidly after administration of MnCl_2 , remaining so for over 3 h and then gradually returning to baseline within 24 h (Figure 3-20A-C). There were no significant difference in T_1 -effective values between 1 h and 4 h after MnCl_2 administration in these organs (Figure 3-20A-C, $p = \text{NS}$ by one-way ANOVA with Bonferroni test). In addition, there were no significant difference in T_1 -effective values between 24 h prior to (-24 h), 24, 48 and 120 h after MnCl_2 administration in these organs (Figure 3-20A-C, $p = \text{NS}$ by one-way ANOVA with Bonferroni test). T_1 -effective values after MnCl_2 administration were decreased by 31 %, 37 % and 33 % in the pancreas, kidney and liver, respectively. While in the muscle, there was no significant change of T_1 -effective values over time following MnCl_2 administration, indicating no obvious Mn^{2+} uptake in these tissues (Figure 3-20D).

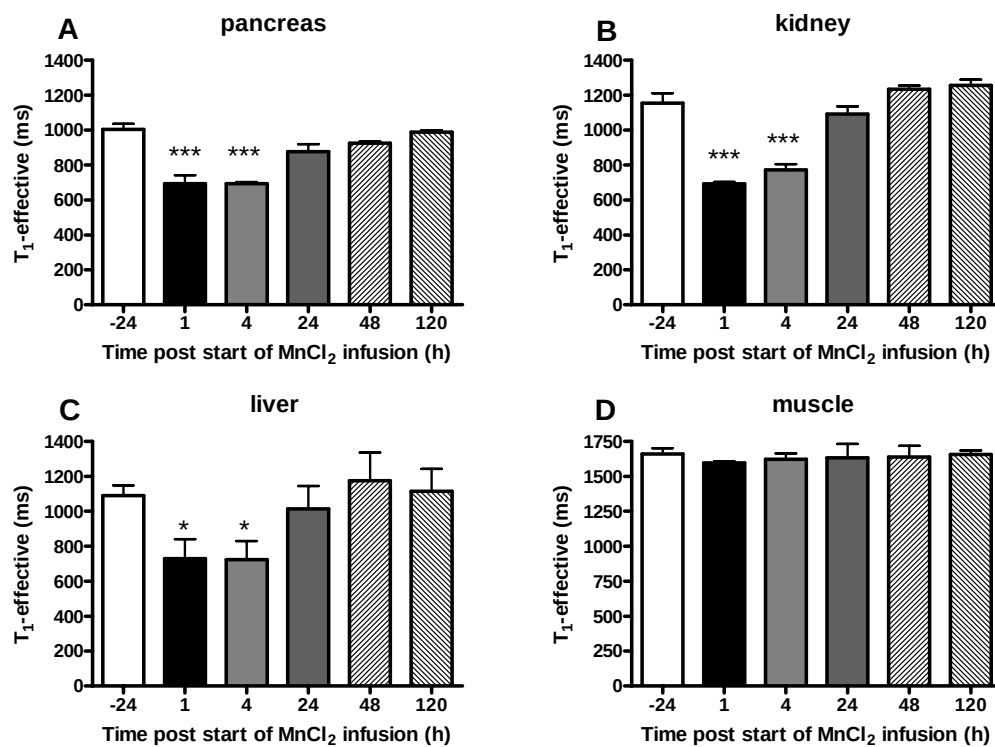


Figure 3-20. Tissue manganese washout in the pancreas (A), kidney (B), liver (C) and muscle (D) 24 h prior to and 1, 4, 24, 48 and 120 h after the start of MnCl₂ infusion, as presented by temporal T₁-effective values. n = 3/group. * = p < 0.05, *** = p < 0.001 by one-way ANOVA with Bonferroni test. Data are presented as mean ± SEM.

3.6 Glucose challenge in healthy mice

3.6.1 Healthy mice

3.6.1.1 Pancreas

3.6.1.1.1 Serial MEMRI

Figure 3-21 shows the SI change over time following MnCl_2 infusion in the glucose-stimulated (glucose, $n = 6$) and vehicle-stimulated (control, water, $n = 6$) pancreas. The SI in the pancreas continued to climb during the period of MnCl_2 infusion and reached a maximum SI value at the end of 30 min MnCl_2 infusion. The maximum enhancement plateau lasted until the end of the experiment (96 min after the start of MnCl_2 infusion). The SI profiles revealed a significantly greater SI in the pancreas of those mice receiving an i.p. injection of glucose compared to controls ($p < 0.05$, GEE). The SI change in glucose injected animals became significantly higher than that of controls at 4 minutes after the i.p. glucose injection and remained so for a further 40 min ($p < 0.05$, Mann-Whitney test). The pancreas of glucose-treated mice showed an increase in steady state signal enhancement compared to that of mice which received vehicle (67.5 % and 54 % in glucose-treated and vehicle-treated animals, respectively).

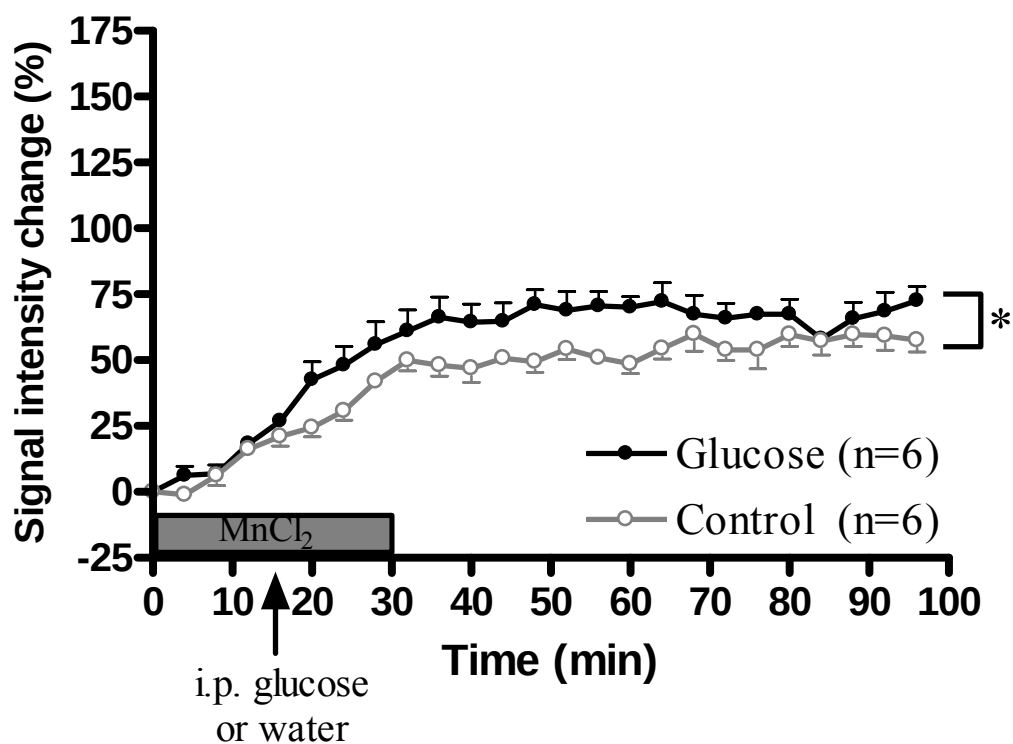


Figure 3-21. The percentage signal intensity change acquired by MP-RAGE following i.v. infusion of MnCl₂ and i.p. bolus injection of glucose or water (control) in C57BL/6 mice (n = 6/group). The grey bar indicates the start and duration of the i.v. MnCl₂ infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or sterile water at 16 min; * = $p < 0.05$ by GEE. Data are presented as mean $\pm$ SEM. All serial MEMRI graph in the glucose challenge experiment are plotted on the same scale.

3.6.1.1.2 T_1 measurements

T_1 -effective values in glucose-stimulated (MnCl_2 + glucose, $n = 8$) and non-stimulated pancreas (MnCl_2 + water, $n = 7$) were performed at 10 min after the end of MnCl_2 infusion and were compared to T_1 -effective values measured in animals receiving no treatment (no MnCl_2 , $n = 6$). Following MnCl_2 infusion and vehicle (water) treatment, T_1 -effective values decreased from 801.3 ± 22.0 ms to 661.0 ± 20.5 ms ($p < 0.001$, Figure 3-22A). With glucose stimulation, T_1 -effective values decreased further to 633.9 ± 20.3 ms. However, there is no significant difference between T_1 -effective values in mice receiving i.p. glucose or water injection after MnCl_2 infusion ($p = 0.37$, Figure 3-22A). R_1 -effective values in the pancreas increased significantly following MnCl_2 infusion ($p < 0.01$, Figure 3-22B). With glucose stimulation, a greater increase in R_1 -effective values was observed ($p < 0.001$, Figure 3-22B). However, no difference in R_1 -effective value was observed between mice receiving i.p. glucose or water injection after MnCl_2 infusion ($p = \text{NS}$, Figure 3-22B).

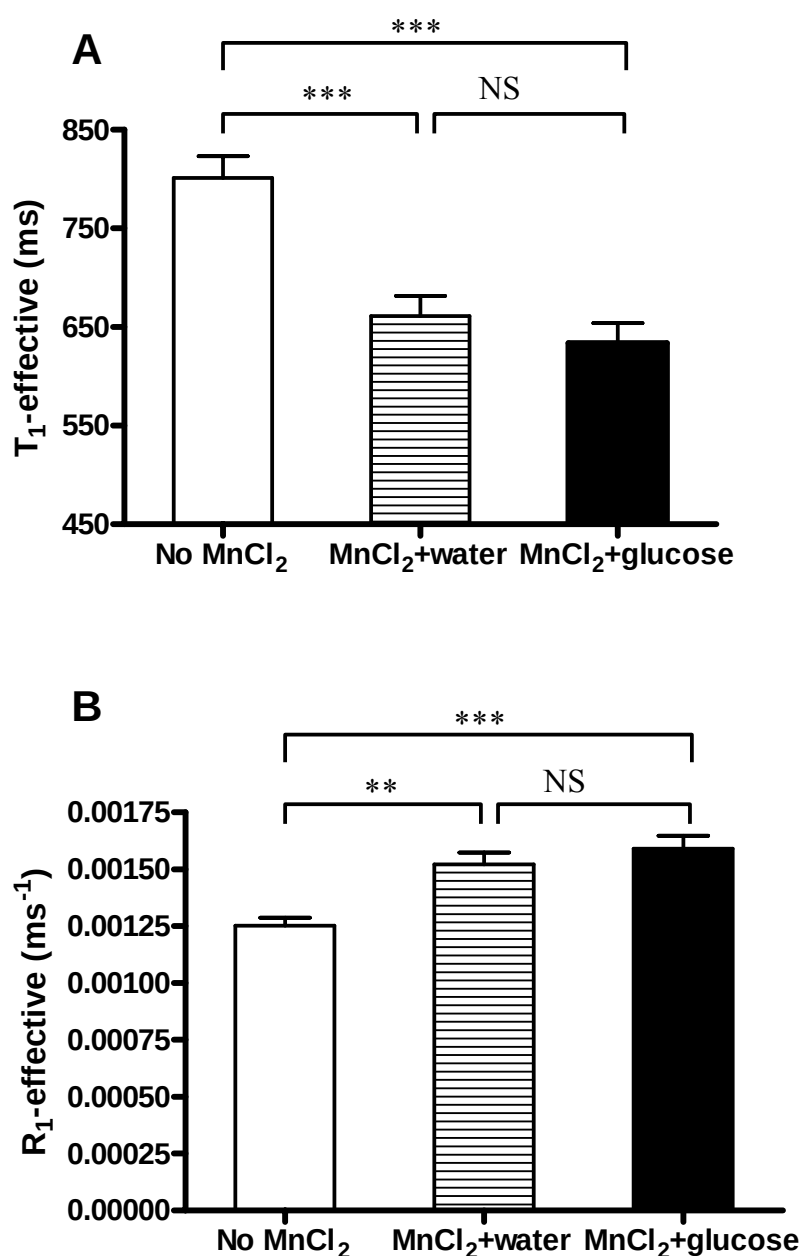


Figure 3-22. T_1 -effective (A) and R_1 -effective (B) values of the pancreas in mice given no treatment (no $MnCl_2$, $n = 6$); i.v. $MnCl_2$ and i.p. water ($MnCl_2$ + water, $n = 7$); and i.v. $MnCl_2$ and i.p. glucose ($MnCl_2$ + glucose, $n = 8$) groups. ** = $p < 0.01$, *** = $p < 0.001$, NS = non-statistically significant by one-way ANOVA with Bonferroni test. Data are presented as mean $\pm$ SEM.

3.6.1.1.3 Tissue [Mn] by ICP-AES

Tissue [Mn] as assessed by ICP-AES in the pancreas rose from 7.74 ± 0.64 $\mu\text{g/g}$ (no MnCl_2 , $n = 3$) to 15.94 ± 0.86 $\mu\text{g/g}$ (MnCl_2 + water, $n = 7$) 66 min after the start of MnCl_2 infusion (Figure 3-23, $p < 0.01$). With glucose stimulation, greater Mn^{2+} uptake was observed in the pancreas, with tissue [Mn] rising to 16.87 ± 1.3 $\mu\text{g/g}$ (MnCl_2 + glucose, $n = 8$; $p < 0.001$). No difference in tissue [Mn] was observed in mice receiving i.p. glucose or water injection after MnCl_2 infusion ($n = 8/\text{group}$, $p = 0.57$).

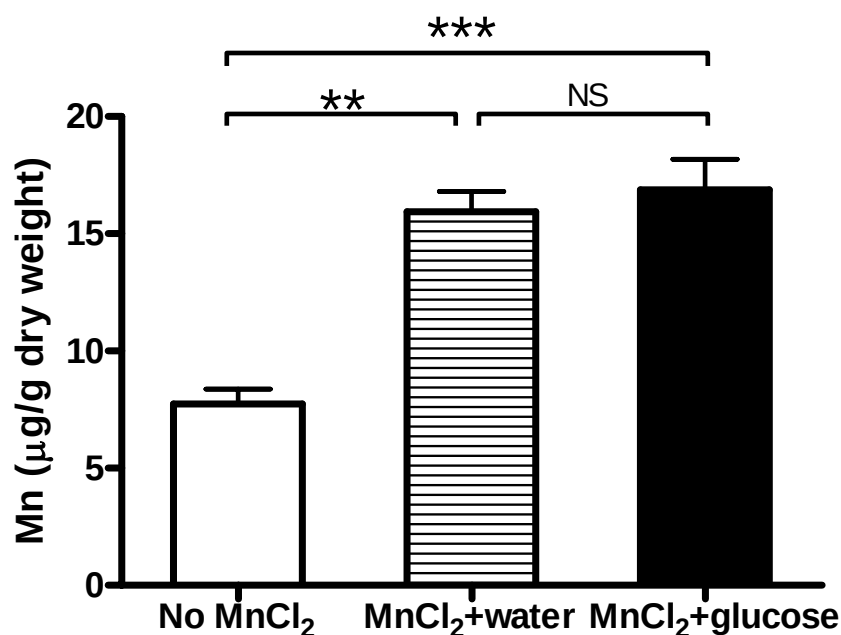


Figure 3-23. Differences in pancreatic manganese content in animals given no treatment (no MnCl_2 , $n = 3$); with i.v. infusion of MnCl_2 and i.p. injection of vehicle (MnCl_2 + water, $n = 7$) or glucose (MnCl_2 + glucose, $n = 8$). Samples were collected at 36 min after the end of MnCl_2 infusion. *** = $p < 0.001$, ** = $p < 0.01$, NS = non-statistically significant by one-way ANOVA with Bonferroni test. Data are presented as mean $\pm$ SEM.

3.6.1.1.4 LA-ICP-MS mapping: a pilot study

The pancreas samples were collected at 66 min after the start of MnCl_2 infusion in mice receiving i.v. MnCl_2 infusion (20 mM, 0.2 mL/h, 0.1 mL) and i.p. injection of glucose or water ($n = 1/\text{group}$). LA-ICP-MS was performed at a resolution of 100 μm in the frozen section of mouse pancreas to illustrate Mn and iron distribution *in vitro*. The quality of H&E stained frozen sections (Figure 3-24) was not good enough to delineate the pancreatic islets but still gave information about the tissue margins of the pancreas and spleen. The Mn map shows a general higher level of Mn in the glucose-stimulated pancreas compared to that of vehicle-treated pancreas (Figure 3-24). Further, the glucose- and vehicle-treated samples analysed by LA-ICP-MS also contained spleen and Mn levels were found to be higher in the pancreas than the spleen in both glucose- and vehicle-treated samples. Mapping of the iron content of the samples showed that the spleen contained much higher amounts of iron than that in pancreata.

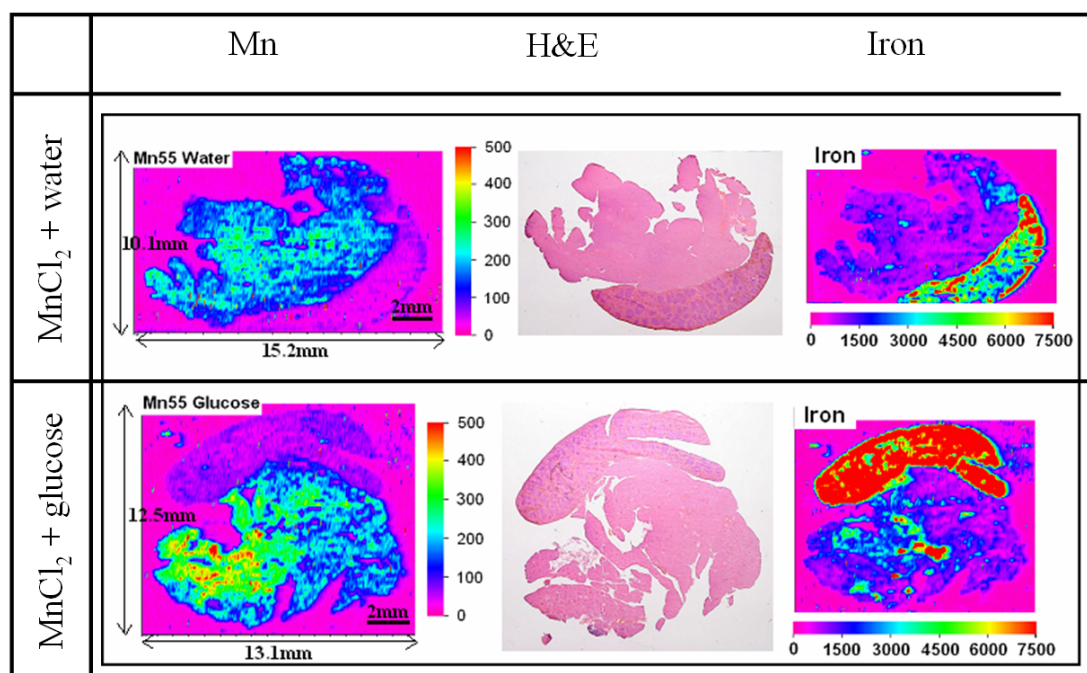


Figure 3-24. Comparison of LA-ICP-MS Mn and Fe maps with corresponding H&E staining of pancreas from C57BL/6 mice given an i.p. dose of glucose and water. Color bar indicates element counts per second.

3.6.1.1.5 XRF mapping: a pilot study

The pancreas samples were collected at 36 min after the start of MnCl_2 infusion in mice receiving i.v. MnCl_2 infusion (2 mM, 0.2 mL/h, 0.1 mL) and i.p. injection of glucose or water ($n = 1/\text{group}$). XRF maps of Mn and Zn were obtained (resolution of $300 \times 300 \mu\text{m}$) of the paraffin sections of mouse pancreas as well as the adjacent H&E stained section. β -cells are known to contain high amount of Zn and correlation between the Zn maps and the presence of islets were observed (Figure 3-25). However, the correlation is unclear due to the much lower resolution of the XRF maps compared to the H&E stained sections. A high degree of correlation was observed between Mn and Zn locations in the glucose-stimulated pancreas, compared to the control pancreas (Figure 3-25). The correspondence is not exact and may arise from not all islets being activated following pancreas stimulation by glucose. Further, mean normalised Mn intensity was higher in the glucose-treated pancreas than the control, 3.17 ± 1.17 vs 1.67 ± 0.63 , respectively.

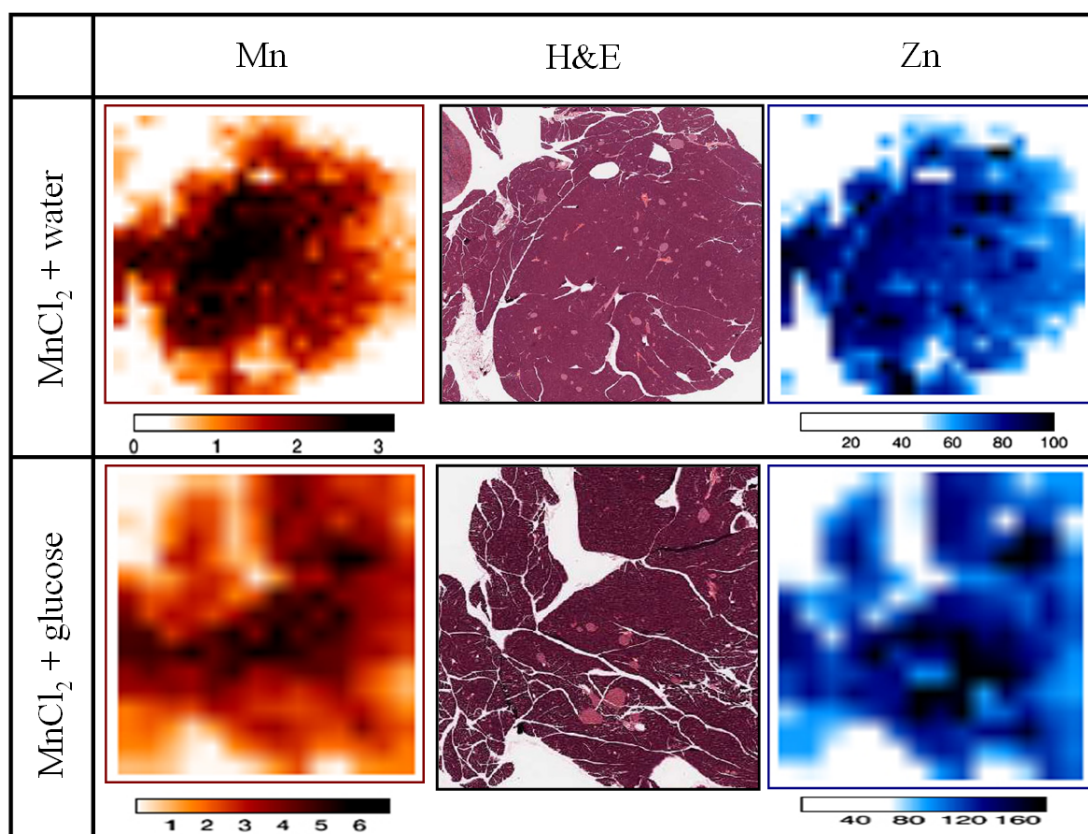


Figure 3-25. Comparison of XRF Mn and Zn maps with corresponding H&E staining of pancreas from C57BL/6 mice give i.p. dose of glucose and water.

3.6.1.2 Skeletal muscle

3.6.1.2.1 Serial MEMRI

MEMRI was further used to measure Mn^{2+} uptake in skeletal muscle. Figure 3-26 shows the effect of glucose on the SI over time in skeletal muscle in healthy mice. Immediately after the administration of MnCl_2 , there was a small peak in the SI profile in the glucose-stimulated and non-stimulated group ($p > 0.05$, Mann-Whitney test). Then, SI increase became faster, reaching a peak 20 min after glucose injection and remaining elevated until the end of the experiment. SI was increased by 20 % in the glucose-stimulated group ($p = 0.006$, GEE; $n = 6/\text{group}$). While in the control group, there was also a small signal enhancement peak in early phase of MnCl_2 infusion, it then returned to baseline and remained so until the end of the experiment.

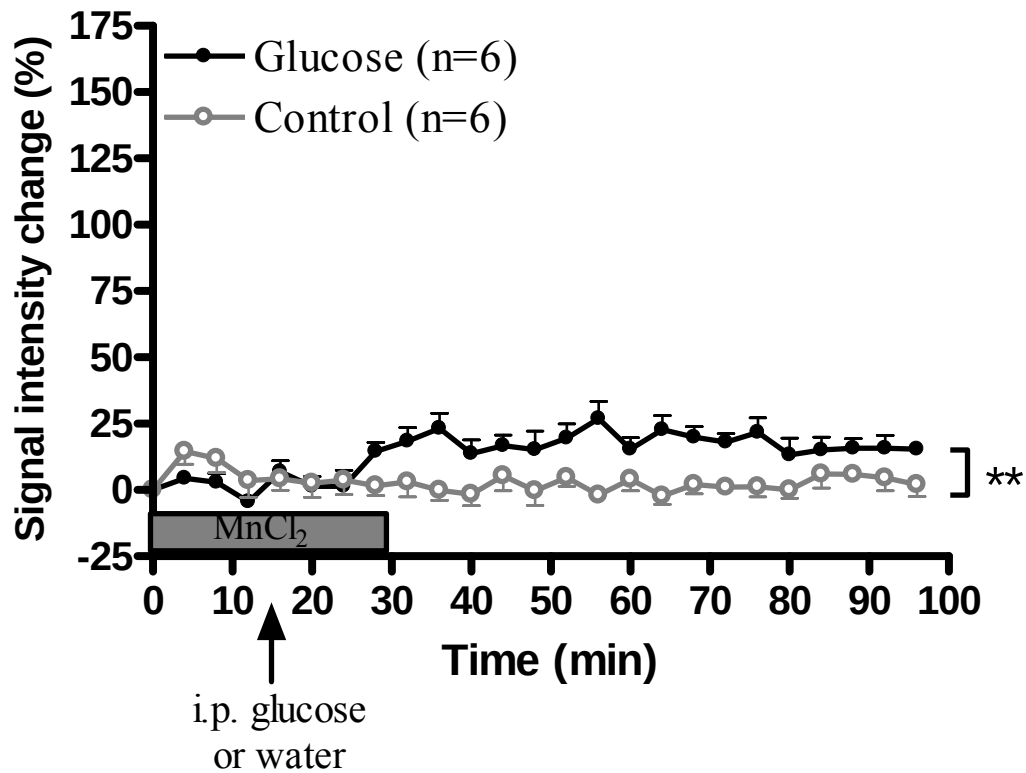


Figure 3-26. Signal intensity profiles in skeletal muscle following MnCl₂ infusion (2 mM, 0.1 mL, 0.2 mL/h) in healthy mice. The grey bar indicates the start and duration of the i.v. MnCl₂ infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or sterile water at 16 min. ** = $p < 0.01$ by GEE. Data are presented as mean $\pm$ SEM. All serial MEMRI graph in the glucose challenge experiment are plotted on the same scale.

3.6.1.2.2 T_1 measurement

In the skeletal muscle, T_1 -effective values were obtained at 40 min after the start of $MnCl_2$ infusion using standard parameters. Both T_1 -effective and R_1 -effective values were not affected by the administration of $MnCl_2$ (no $MnCl_2$ vs. $MnCl_2$ + water, Figure 3-27, $p > 0.05$ by one-way ANOVA). There were generally lower T_1 -effective and higher R_1 -effective values in the muscle following glucose stimulation (at 16 min after the start of $MnCl_2$ infusion). However, the difference did not reach statistically significant ($MnCl_2$ + glucose vs. $MnCl_2$ + water, Figure 3-27, $p > 0.05$ by one-way ANOVA)

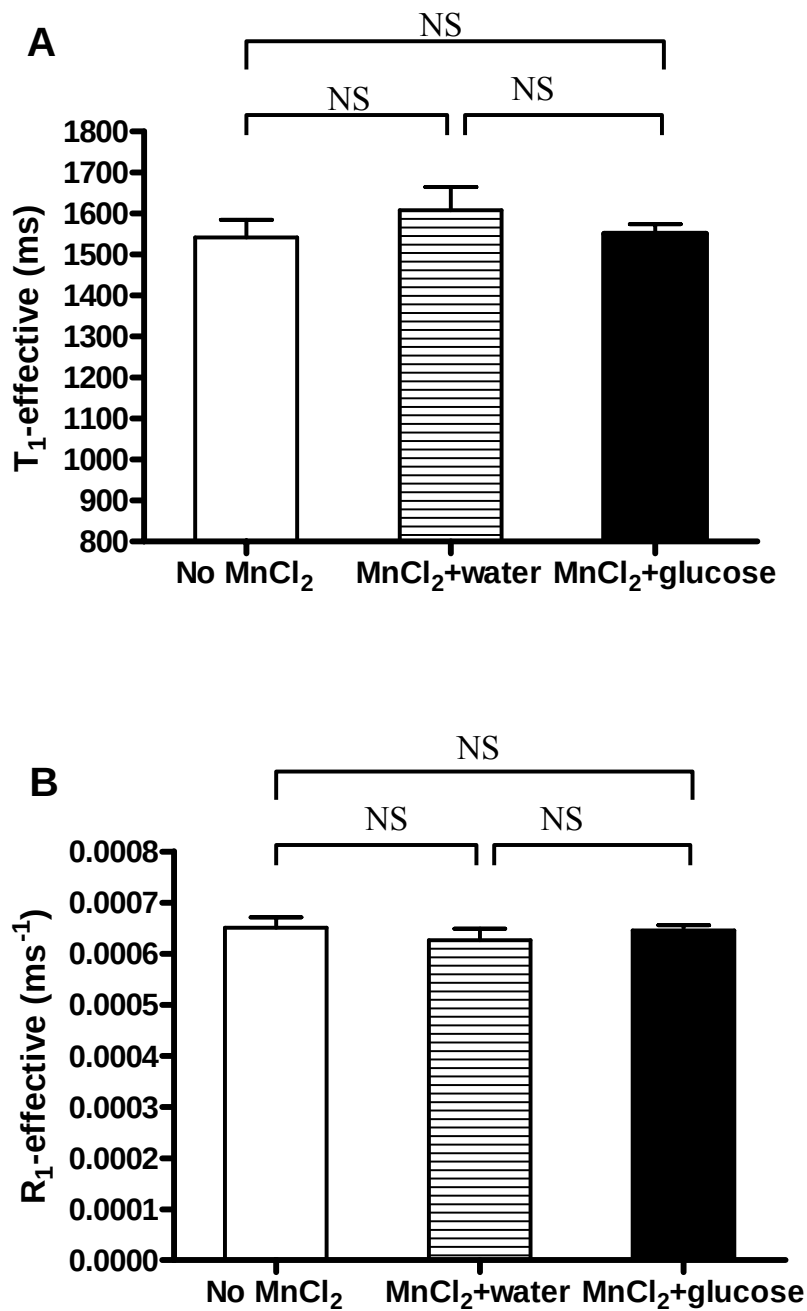


Figure 3-27 T_1 -effective (A) and R_1 -effective (B) values of skeletal muscle obtained by MP-RAGE MRI (TD = 2 s) in animals given no treatment (no $MnCl_2$, $n = 6$), i.v. $MnCl_2$ and i.p. water ($MnCl_2$ + water, $n = 7$) and i.v. $MnCl_2$ and i.p. glucose ($MnCl_2$ + glucose, $n = 8$). NS = $p > 0.05$ by one-way ANOVA with Bonferroni test. Data are presented as mean $\pm$ SEM.

Since skeletal muscle has longer T_1 -effective values compared to the rest organs of interest and a longer TD (3 s) was also required to increase TR_0 . T_1 -effective values of skeletal muscle were obtained 50-110 min after the start of $MnCl_2$ infusion and glucose stimulation was performed at 16 min after the start of $MnCl_2$ infusion. Both T_1 -effective and R_1 -effective values were not affected by the stimulation of glucose following $MnCl_2$ infusion (Figure 3-28A and B, $p > 0.05$ by t-test).

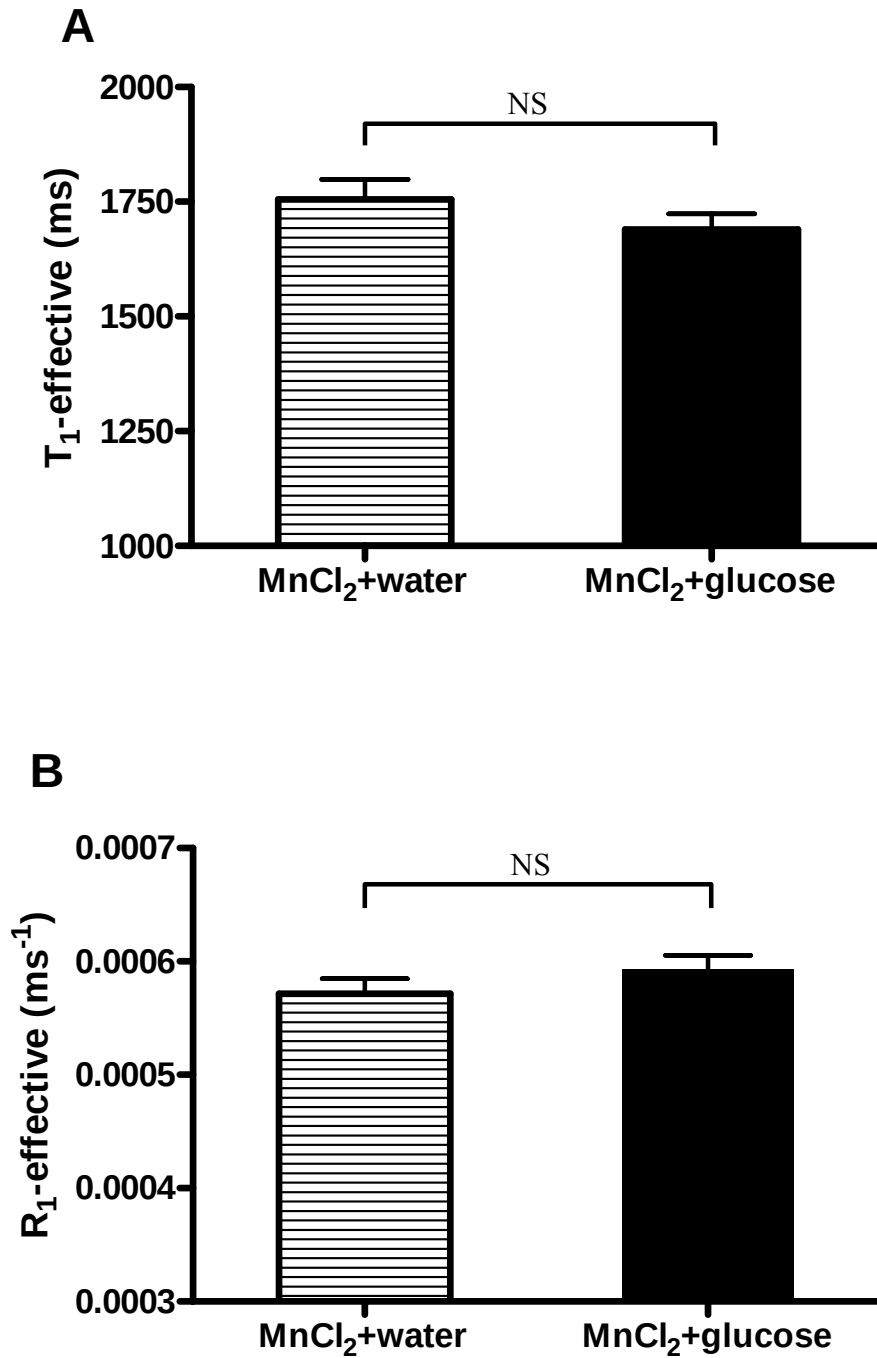


Figure 3-28. T_1 -effective and R_1 -effective values of skeletal muscle obtained by MP-RAGE MRI (TD = 3 s) in mice given MnCl₂ and i.p. glucose (MnCl₂ + glucose, n = 6) or water (MnCl₂ + water, n = 6). NS = $p > 0.05$ by t-test. Data are presented as mean $\pm$ SEM.

3.6.1.2.3 Tissue [Mn] by ICP-AES

Tissue [Mn] obtained at 66 min after the start of MnCl_2 infusion rose from 0.89 ± 0.02 $\mu\text{g/g}$ (no MnCl_2 , $n = 4$) to 1.17 ± 0.02 $\mu\text{g/g}$ (MnCl_2 + water, $n = 10$) ($p < 0.01$). With glucose stimulation, tissue [Mn] rose further to 1.23 ± 0.06 $\mu\text{g/g}$ (MnCl_2 + glucose, $n = 10$), however, this did not reach significance (Figure 3-29, $p > 0.05$ by one-way ANOVA with Bonferroni test).

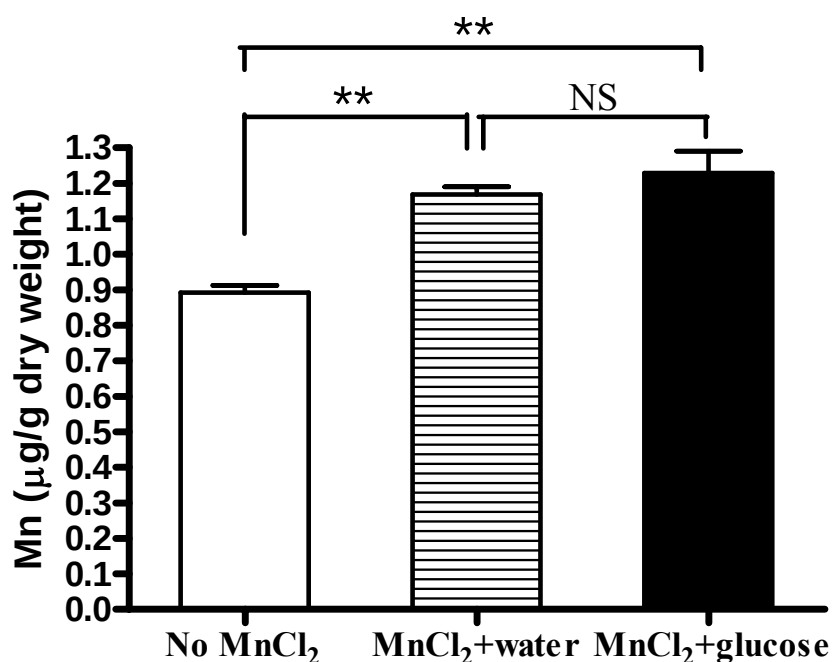


Figure 3-29. Differences in manganese content in skeletal muscle in animals given no treatment (no MnCl_2 , $n = 4$); with i.v. infusion of MnCl_2 and i.p. injection of vehicle (MnCl_2 + water, $n = 10$) or glucose (MnCl_2 + glucose, $n = 10$). Samples were collected 36 min after the end of MnCl_2 infusion. ** = $p < 0.01$, NS = non-statistically significant by one-way ANOVA with Bonferroni test. Data are presented as mean $\pm$ SEM.

3.6.1.3 Liver

3.6.1.3.1 Serial MEMRI

Figure 3-30 shows the SI change over time following MnCl_2 infusion in the glucose-stimulated (glucose, $n = 6$) and vehicle-treated (control, $n = 6$) liver in healthy mice. SI in the liver continued to climb during the period of MnCl_2 infusion and reached a maximum SI value at the end of the MnCl_2 infusion, which plateaued and lasted until the end of the experiment (66 min after the end of MnCl_2 infusion). The overall SI profiles revealed no significant change in SI in the liver receiving an i.p. injection of glucose compared to controls in healthy mice ($p = 0.19$, GEE; Figure 3-30).

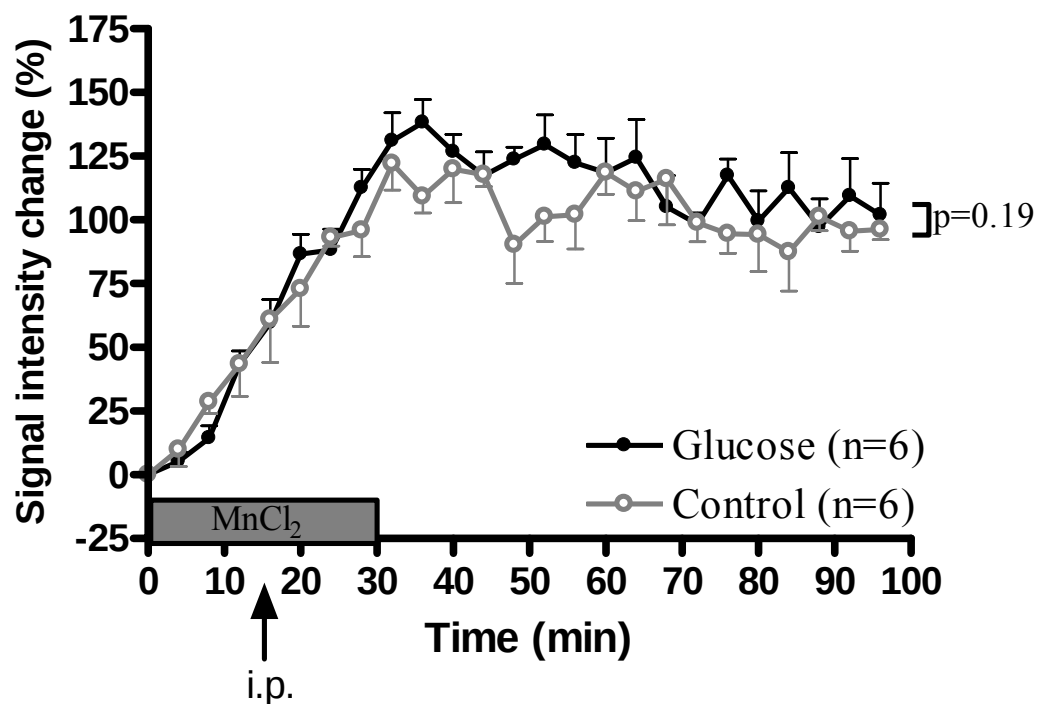


Figure 3-30. Signal intensity profiles in the mouse liver following MnCl_2 infusion (2 mM, 0.1 mL, 0.2 mL/h) in healthy mice receiving i.p. glucose (glucose) or vehicle (control). The grey bar indicates the start and duration of the i.v. MnCl_2 infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or sterile water at 16 min. Signal intensity profiles were tested by GEE. All serial MEMRI graph in the glucose challenge experiment are plotted on the same scale.

3.6.1.3.2 T_1 measurement

T_1 -effective measurement was performed at 40 min after the start of $MnCl_2$ (Figure 3-31A). In the non-treated liver, the T_1 -effective value was 1160.0 ± 28.5 ms (no $MnCl_2$, $n = 6$). After $MnCl_2$ administration, T_1 -effective values were decreased to 731.3 ± 43.6 ms in the glucose-stimulated liver ($MnCl_2$ + glucose, $n = 8$) and 762.5 ± 69.5 ms in the vehicle-stimulated liver ($MnCl_2$ + water, $n = 7$). Both T_1 -effective and R_1 -effective values were not affected by the stimulation of glucose following $MnCl_2$ infusion (Figure 3-31, $p > 0.05$ by one-way ANOVA with Bonferroni test).

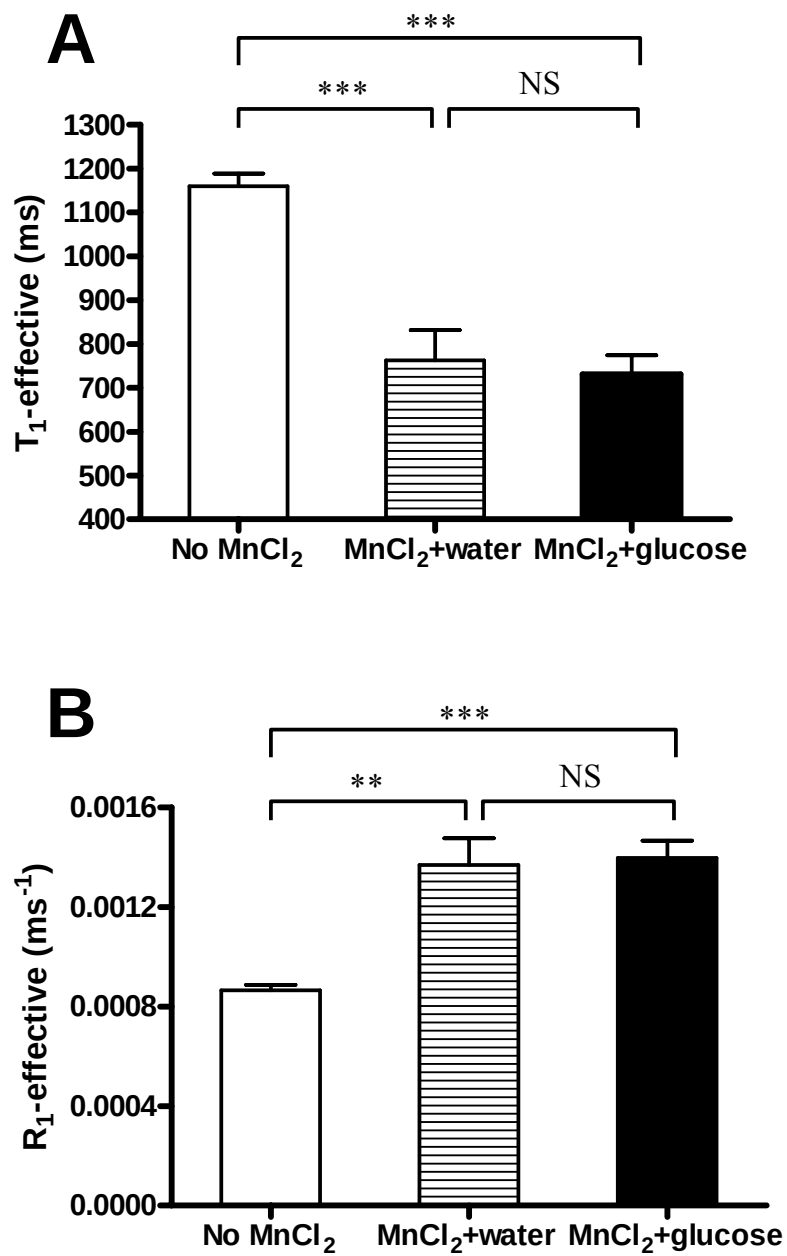


Figure 3-31. T_1 -effective (A) and R_1 -effective (B) values in the mouse liver given no treatment (no $MnCl_2$, $n = 6$); i.v. $MnCl_2$ and i.p. water ($MnCl_2 + water$, $n = 7$); and i.v. $MnCl_2$ and i.p. glucose ($MnCl_2 + glucose$, $n = 8$) groups. ** = $p < 0.01$, *** = $p < 0.01$, NS = non-statistically significant by one-way ANOVA with Bonferroni test. Data are presented as mean $\pm$ SEM.

3.6.1.3.3 Tissue [Mn] by ICP-AES

Tissue [Mn] in the liver was 5.3 ± 0.5 $\mu\text{g/g}$ (no MnCl_2 , $n = 3$) in animals given no treatment (Figure 3-32). At 66 min after the start of MnCl_2 infusion, tissue [Mn] in the liver rose to 11.1 ± 0.4 $\mu\text{g/g}$ (MnCl_2 + water, $n = 7$) in vehicle-stimulated mice and 10.7 ± 0.5 $\mu\text{g/g}$ (MnCl_2 + glucose, $n = 8$) in glucose-stimulated mice (Figure 3-32). However, no difference in tissue [Mn] was observed in mice receiving i.p. glucose or vehicle treatment after MnCl_2 infusion (Figure 3-32, $p > 0.05$ by one-way ANOVA with Bonferroni test).

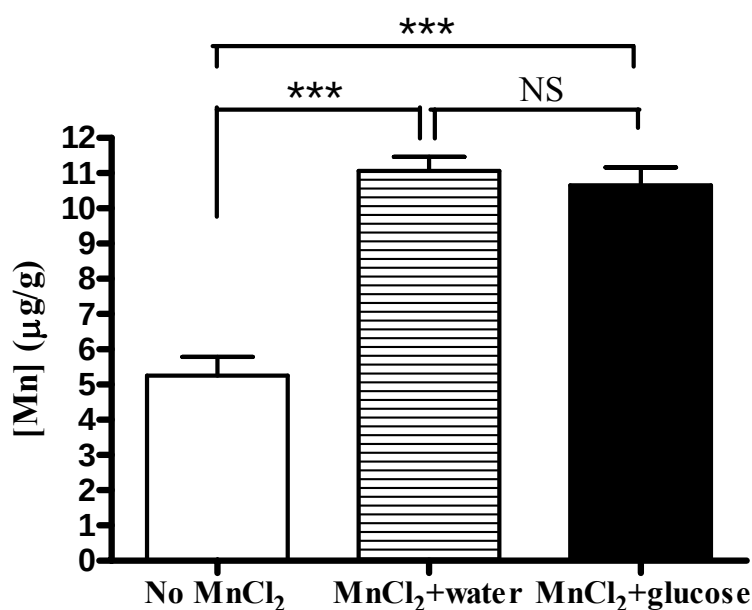


Figure 3-32. Differences in manganese content in the mouse liver given no treatment (no MnCl_2 , $n = 3$), with i.v. infusion of MnCl_2 and i.p. injection of vehicle (MnCl_2 + water, $n = 7$) or glucose (MnCl_2 + glucose, $n = 8$). Samples were collected at 66 min after the start of MnCl_2 infusion. *** = $p < 0.001$, NS = non-statistically significant by one-way ANOVA with Bonferroni test. Data are presented as mean $\pm$ SEM

3.6.1.4 Epididymal fat (white adipose tissue)

3.6.1.4.1 Serial MEMRI

As mentioned previously (see section 3.4.1), the SI measurements could not be obtained from the same ROI in the epididymal fat throughout a given scan set. After adjustment of ROIs, SI changes over time following MnCl_2 infusion in the glucose-stimulated (glucose, $n = 6$) and vehicle-treated (control, $n = 6$) mice are shown in Figure 3-33. A significant difference in the SI profiles prior to administration of glucose and vehicle (Figure 3-33, $p = 0.04$ by GEE) can be observed. This probably arises from the inconsistency in ROI placement. With glucose stimulation, SI profiles was significantly low compared to control group (Figure 3-33, $p = 0.01$ by GEE). However, the data is not reliable as a significant difference in SI was found prior to the application of i.p. stimuli when both groups were treated with only MnCl_2 .

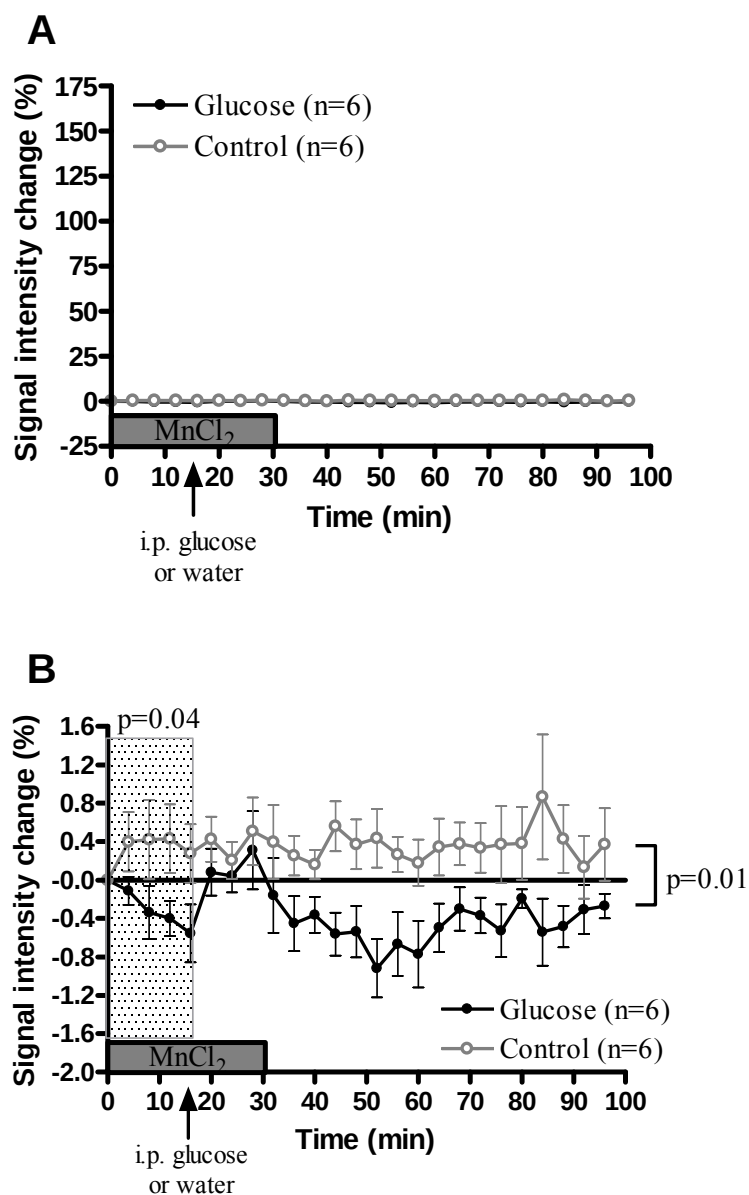


Figure 3-33. Signal intensity profiles in the mouse epididymal fat following MnCl₂ infusion (2 mM, 0.1 mL, 0.2 mL/h) in healthy mice receiving i.p glucose (glucose, n = 6) or vehicle (control, n = 6). Both figure (A) and (B) were plotted using the same data but with different scales on the vertical (y) axis. The grey bar indicates the start and duration of the i.v. MnCl₂ infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or sterile water at 16 min. Signal intensity profiles were tested by GEE.

3.6.1.4.2 T_1 measurement

T_1 -effective values measurements were obtained from the epididymal fat of glucose-stimulated (glucose, $n = 8$) and non-stimulated mice (control, $n = 7$) at 40 min after the start of $MnCl_2$ infusion and were compared to those from animals not treated with $MnCl_2$, glucose or water (no $MnCl_2$, $n = 6$). All ROIs were selected from the same place for each animal. Following $MnCl_2$ infusion and water injection, T_1 -effective values decreased from 231.4 ± 26.6 ms to 123.0 ± 27.5 ms ($p < 0.05$, Figure 3-34A). With glucose stimulation, T_1 -effective values were 166.8 ± 24.9 ms and not significantly different from the epididymal fat of animals having no treatment ($p > 0.05$, Figure 3-34A). No difference was observed in T_1 -effective values in mice receiving i.p. glucose or water injection after $MnCl_2$ infusion ($p > 0.05$, Figure 3-34A). R_1 -effective values in the epididymal fat increased significantly following $MnCl_2$ infusion ($p < 0.01$, Figure 3-34B). However, no difference in R_1 -effective value was observed between mice receiving i.p. glucose or water injection after $MnCl_2$ infusion ($p > 0.05$, Figure 3-34B).

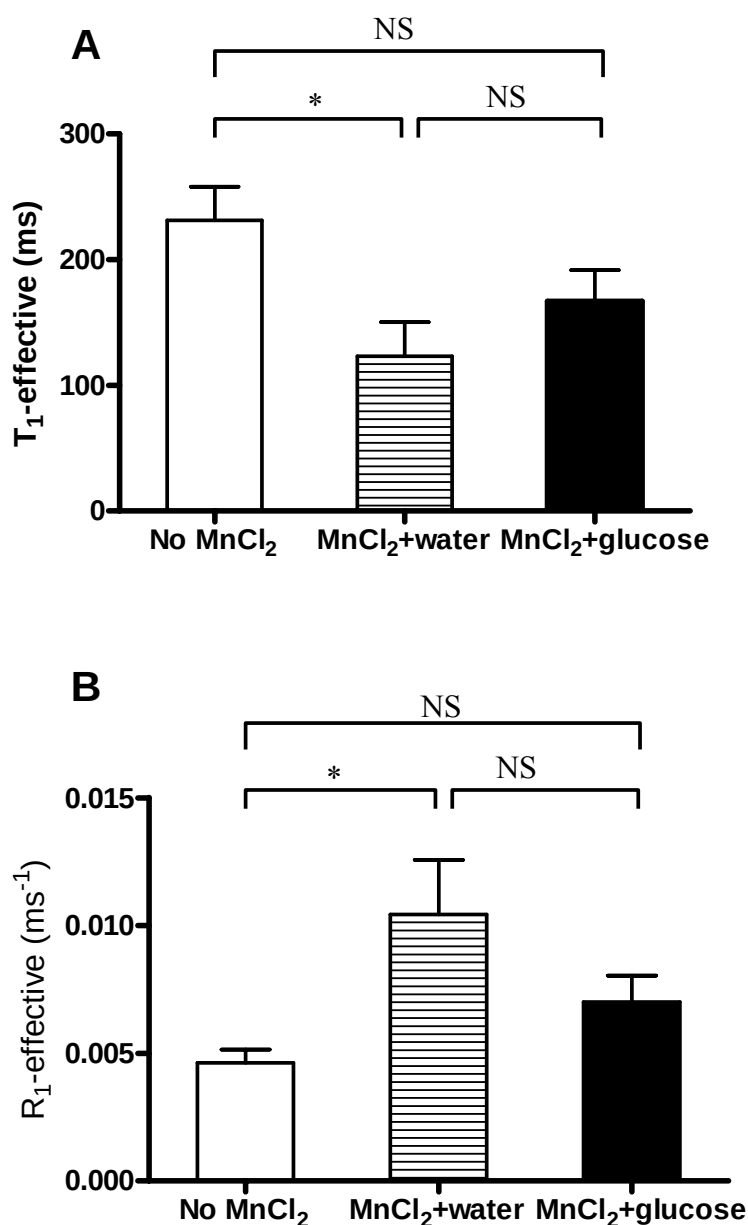


Figure 3-34. T_1 -effective (A) and R_1 -effective (B) values of the epididymal fat in mice given no treatment (no $MnCl_2$, $n = 6$), i.v. $MnCl_2$ and i.p. water ($MnCl_2$ + water, $n = 7$) and i.v. $MnCl_2$ and i.p. glucose ($MnCl_2$ + glucose, $n = 8$) groups. * = $p < 0.05$, NS = non-statistically significant by one-way ANOVA with Bonferroni test. Data are presented as mean $\pm$ SEM.

3.6.1.4.3 Tissue [Mn] by ICP-AES

Even after MnCl_2 infusion, tissue [Mn] in the epididymal fat was below the detection limit in 5 out of 15 samples and therefore, only the available data were analysed here. Tissue [Mn] in the epididymal fat appeared to decline from $0.16 \pm 0.05 \mu\text{g/g}$ (MnCl_2 + water, $n = 5$) to $0.09 \pm 0.05 \mu\text{g/g}$ (MnCl_2 + glucose, $n = 5$) following glucose stimulation although not significantly (Figure 3-35, $p > 0.05$).

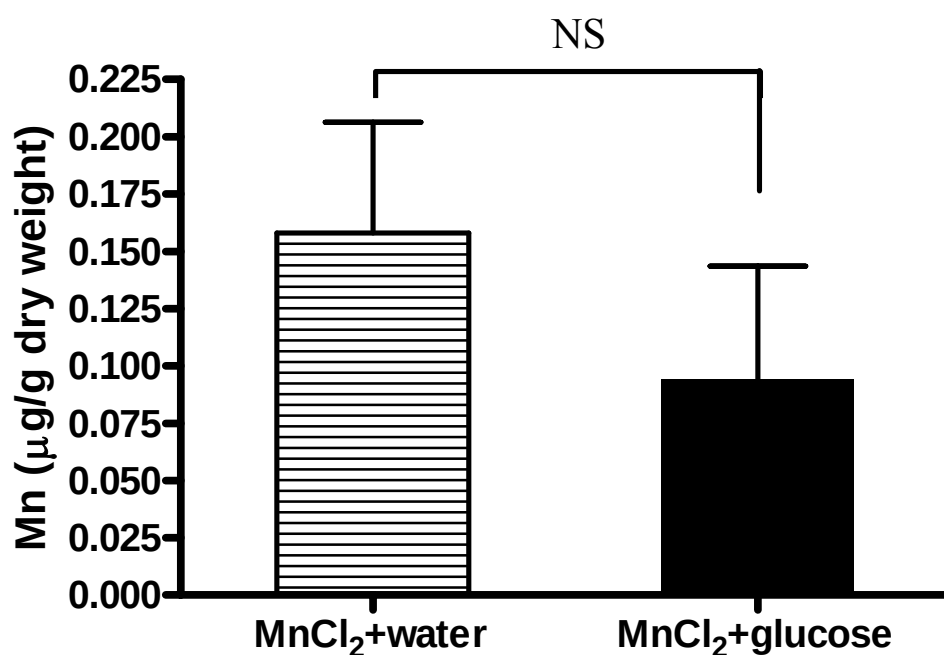


Figure 3-35. Differences in manganese content in the mouse epididymal fat given MnCl_2 and i.p. glucose (MnCl_2 + glucose, $n = 5$) or water (MnCl_2 + water, $n = 5$). NS = non-statistically significant by t-test. Data are presented as mean $\pm$ SEM.

3.6.1.5 Interscapular fat (brown adipose tissue)

3.6.1.5.1 Serial MEMRI

The interscapular fat contains a mixture of WAT and BAT. The interscapular fat is a triangular-shaped fat pad between the scapulae and ROIs were placed here. In this experiment, the interscapular fat was discerned in 8 out of 12 animals and so only this data was analysed. Therefore, only the available data were analysed. Figure 3-36 shows the SI change over time following MnCl_2 infusion in the glucose-stimulated (glucose, $n = 5$) and vehicle-treated (control, $n = 3$) mice. SI rose following MnCl_2 infusion and immediately reached a maximum enhancement plateau at 4 min after the start of infusion. The maximum enhancement plateau lasted until the latest time point of the experiment (~ 96 min after the start of MnCl_2 infusion). No significant change in SI profiles was observed between glucose-treated and control mice (Figure 3-36, $p = 0.59$ by GEE).

However, the SI in the interscapular fat could not be measured from the same ROI throughout a given serial MEMRI experiment. Moreover, dose-dependent T_1 measurement results in section 4.4.2 show that this MP-RAGE method may be unreliable in estimating T_1 values in the interscapular fat. Therefore, SI profiles in the interscapular fat were not analysed further.

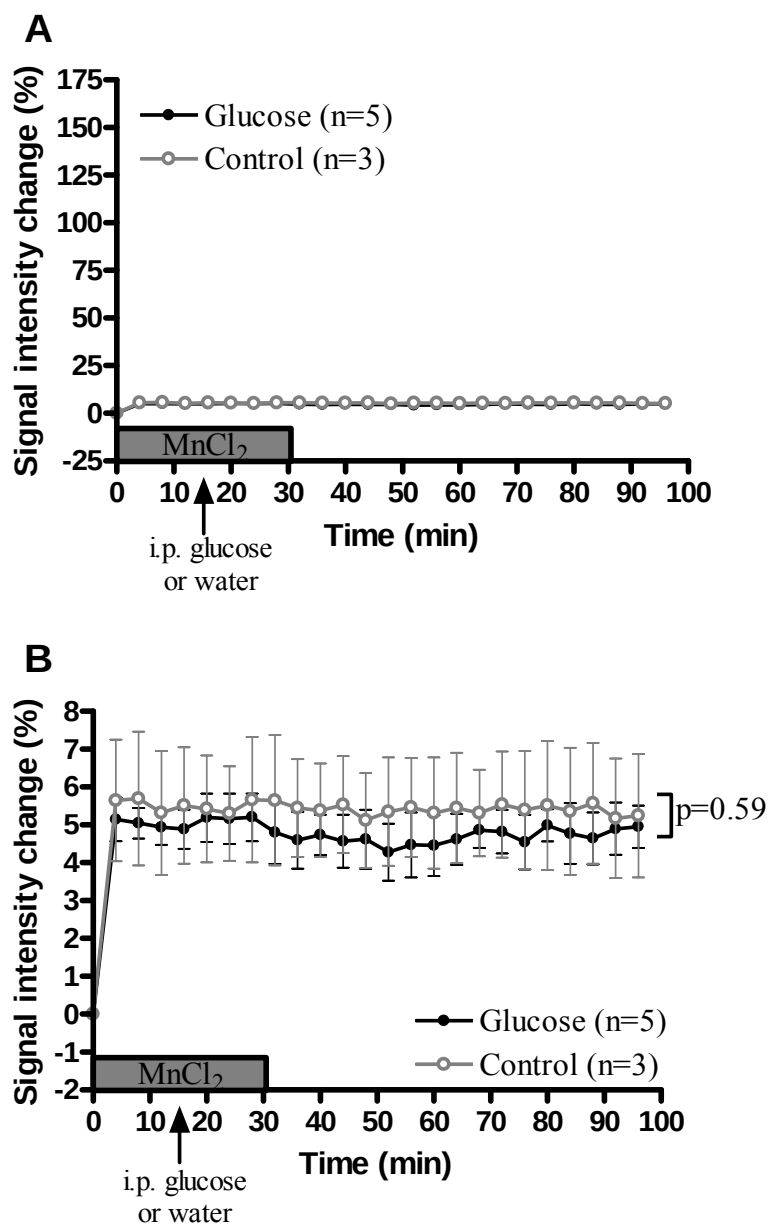


Figure 3-36. Signal intensity profiles in the interscapular fat following MnCl₂ infusion (2 mM, 0.1 mL, 0.2 mL/h) in mice receiving i.p glucose (glucose, n = 3) or vehicle (control, n = 5). Both figure (A) and (B) were plotted using the same data but with different scales on the vertical (y) axis. The grey bar indicates the start and duration of the i.v. MnCl₂ infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or sterile water at 16 min. Signal intensity profiles were tested by GEE.

3.6.1.6 Effect of MnCl_2 infusion on blood glucose levels

Serial blood glucose measurements were performed in anesthetized mice following overnight fasting. Blood samples were taken from the tail vein at the start and at 32, 36, 40, 44, 48, 52, 56 and 60 min after the start of the experiment. Tail vein sampling was not performed in the first 30 min of the experiment when MnCl_2 infusion was performed in some of the animals.

The initial mean blood glucose level of all animals at the start of this experiment was 5.6 ± 0.3 mM (Figure 3-37, $n = 23$). There was a general increase in blood glucose levels over time in animals receiving no treatment ($n = 5$) and i.p. water injection ($n = 4$ and 5 in animals with and without MnCl_2 infusion, respectively), but the increase was not significant (Figure 3-37, $p = \text{NS}$ by one-way ANOVA with Bonferroni test). In Figure 3-37, glucose i.p. injection resulted in a significant increase in blood glucose levels of up to 20 mM in C57BL/6 mice with and without MnCl_2 infusion ($n = 4$ and 5, respectively; $p < 0.001$ by one-way ANOVA with Bonferroni test) compared to controls.

The peak blood glucose levels were defined as the average blood glucose levels acquired at 32-60 min after the start of MnCl_2 infusion or at the corresponding time. There was no significant difference in the mean peak blood glucose levels in animals receiving no treatment; i.p. water treatment; and MnCl_2 infusion with i.p. water (Figure 3-37, $p = \text{NS}$ by one-way ANOVA with Bonferroni test). There was also no significant difference in the mean peak blood glucose levels of glucose-treated animals with without MnCl_2 infusion (Figure 3-37, $p = \text{NS}$ by t-test). In summary, intravenous

infusion of MnCl_2 appeared to have no effect upon the blood glucose levels in control and that induced by i.p. injection of glucose.

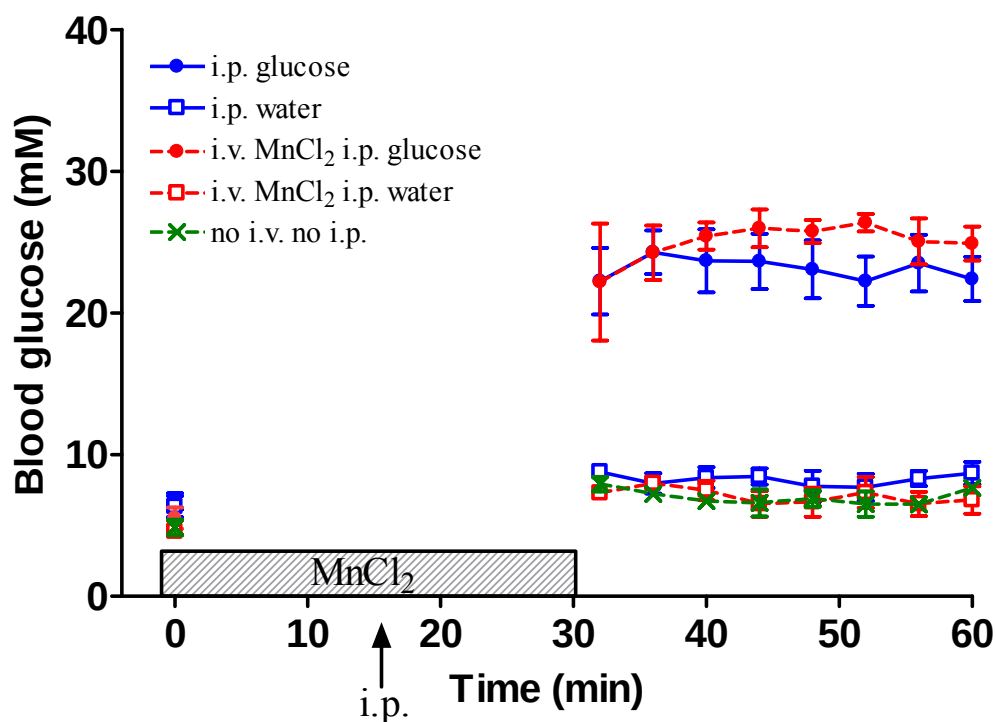


Figure 3-37. Blood glucose levels following i.p. administration of glucose, water or non-i.p. administration in non- MnCl_2 and MnCl_2 infused mice. The grey bar indicates the start and duration of the i.v. MnCl_2 infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or water ($n = 4-5/\text{group}$). Data are presented as mean $\pm$ SEM.

3.6.2 Diabetic mouse model

3.6.2.1 Pancreas

3.6.2.1.1 Serial MEMRI

Figure 3-38 shows the pancreatic SI changes over time following MnCl_2 infusion in the glucose-stimulated (STZ + glucose) and vehicle-treated (STZ - control) diabetic mice ($n = 4/\text{group}$). SI rose during the period of MnCl_2 infusion and reached a maximal value at ~ 40 min. The maximum enhancement plateau lasted until the end of the experiment, 96 min after the start of infusion. The SI profiles in the pancreas were not significantly different between glucose-stimulated and control groups in the diabetic mice ($p = 0.78$, GEE).

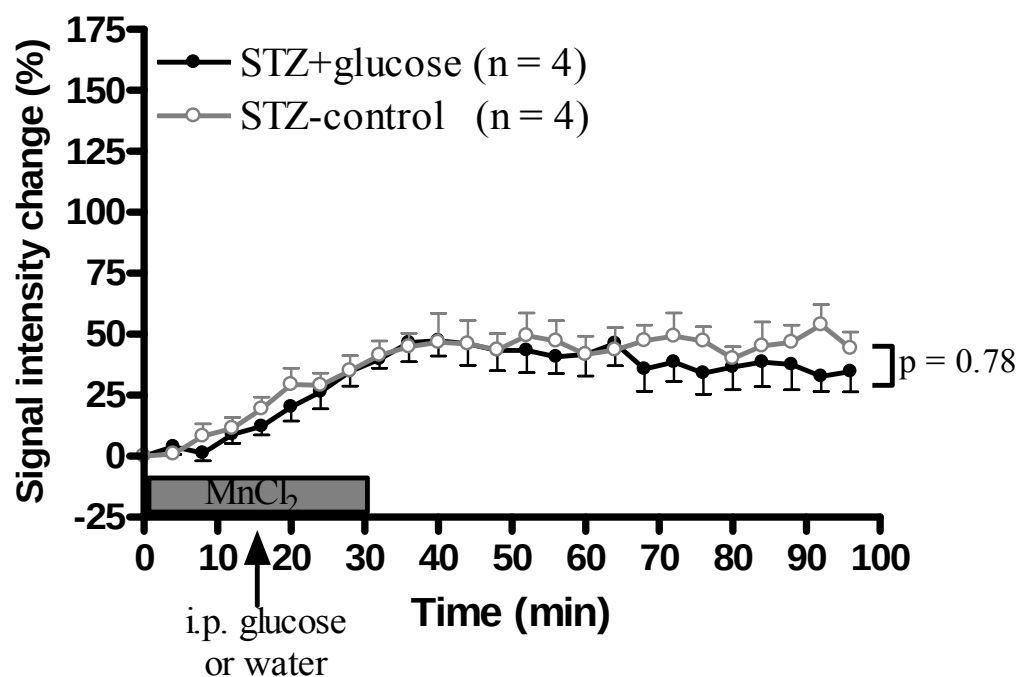


Figure 3-38. The percentage signal intensity change in the pancreas following i.v. infusion of MnCl₂ and i.p. bolus injection of glucose (STZ + glucose, n = 4) or vehicle (STZ - control, n = 4) in STZ-induced diabetic mice. The grey bar indicates the start and duration of the i.v. MnCl₂ infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or sterile water at 16 min. Signal intensity profiles were tested by GEE. Data are presented as mean $\pm$ SEM. All serial MEMRI graph in the glucose challenge experiment are plotted on the same scale.

3.6.2.2 Skeletal muscle

3.6.2.2.1 Serial MEMRI

In STZ-treated diabetic mice (Figure 3-39), there was a similar signal enhancement pattern in skeletal muscle as in healthy mice. There was no significant difference in SI in the glucose-stimulated (STZ + glucose, $n = 4$) and control groups (STZ – control, $n = 4$) following $MnCl_2$ infusion (Figure 3-39, $p = 0.16$ by GEE).

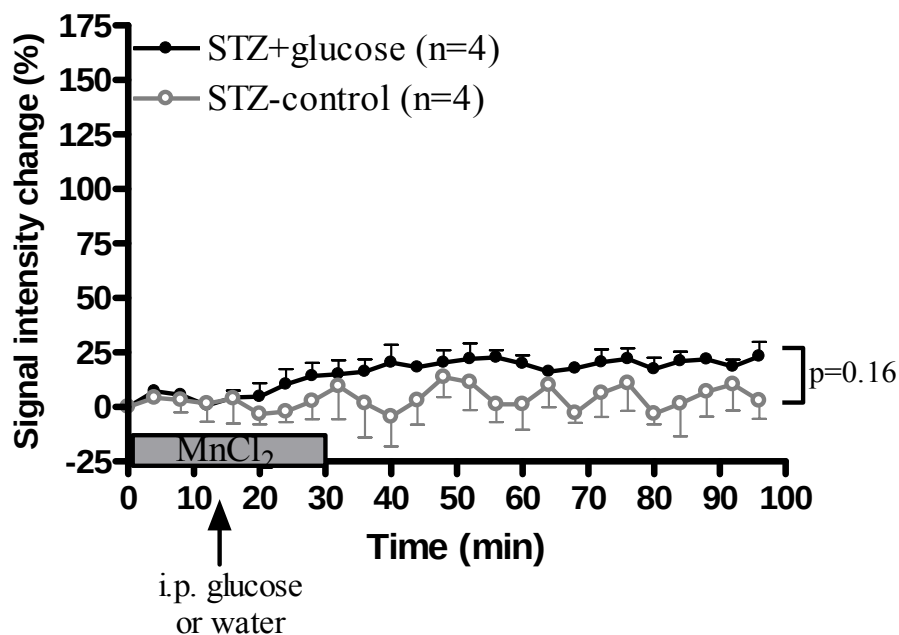


Figure 3-39. The percentage signal intensity change in skeletal muscle following i.v. infusion of $MnCl_2$ and i.p. bolus injection of glucose (STZ + glucose, $n = 4$) or vehicle (STZ - control, $n = 4$) in STZ-induced diabetic mice. The grey bar indicates the start and duration of the i.v. $MnCl_2$ infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or sterile water at 16 min. Signal intensity profiles were tested by GEE. Data are presented as mean $\pm$ SEM. All serial MEMRI graph in the glucose challenge experiment are plotted on the same scale.

3.6.2.3 Liver

3.6.2.3.1 Serial MEMRI

Figure 3-40 shows the SI change over time following MnCl_2 infusion in the glucose-stimulated and vehicle-stimulated (water, control) liver in STZ-treated mice. SI in the liver rose during the period of MnCl_2 infusion and reached maximum SI value after 30 min. The maximum enhancement plateau lasted until the end of the experiment, 96 min after the start of infusion. The overall SI profiles revealed no significant change in the liver of diabetic mice receiving an i.p. injection of glucose compared to those receiving i.p. injection of the vehicle ($p = 0.82$, GEE; $n = 4/\text{group}$; Figure 3-40).

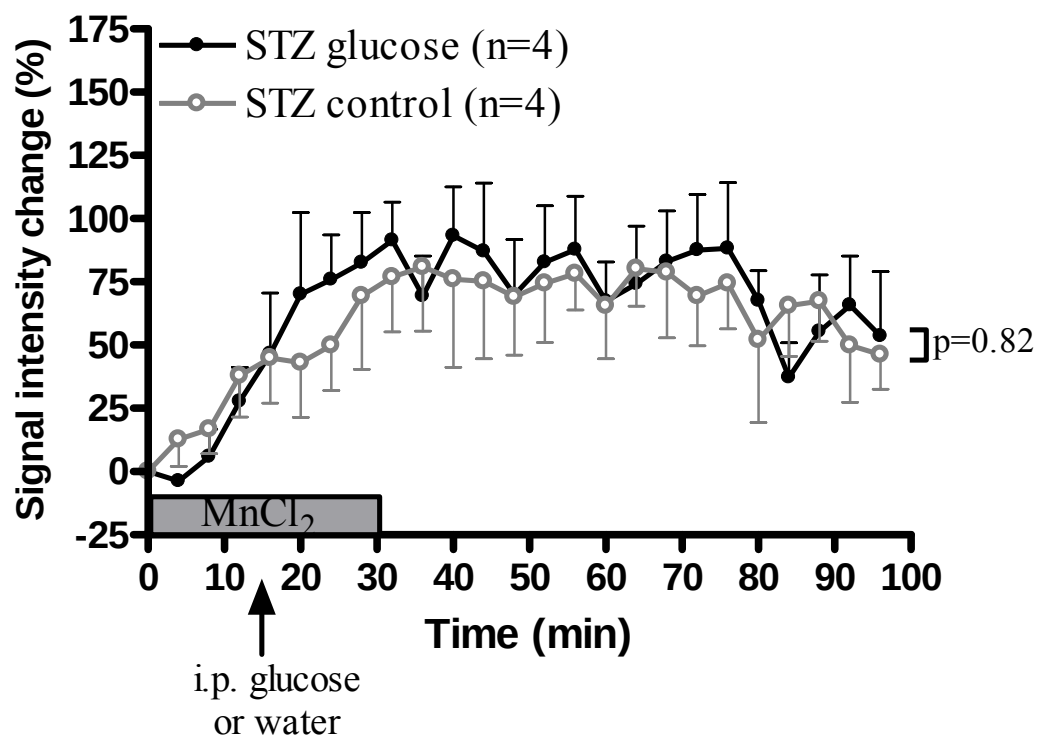


Figure 3-40. The percentage signal intensity change in the livers following i.v. infusion of MnCl₂ and i.p. bolus injection of glucose (STZ glucose, n = 4) or vehicle (STZ control, n = 4) in STZ-induced diabetic mice. The grey bar indicates the start and duration of the i.v. MnCl₂ infusion. The arrow indicates the timing of the bolus i.p. injection of glucose or sterile water at 16 min. Signal intensity profiles were tested by GEE. All serial MEMRI graph in the glucose challenge experiment are plotted on the same scale.

3.7 Histology of the pancreas

3.7.1 The effect of MnCl_2 infusion on islet morphology

In this study, mouse pancreatic sections were probed with insulin or glucagon antibody. Insulin-positive β -cells and glucagon-positive α -cells in pancreatic sections were stained brown by insulin and glucagon staining, respectively (Figure 3-41). The islets of control mice (no MnCl_2) consisted mostly of insulin-positive cells while glucagon-positive α -cells were much less abundant (Figure 3-41A-C). Infusion of MnCl_2 (2 mM, 0.1 mL) had no significant effect on islet morphology and cell numbers, as determined by H&E stain and immunostaining (Figure 3-41A-C versus D-F), indicating no sign of acute toxicity.

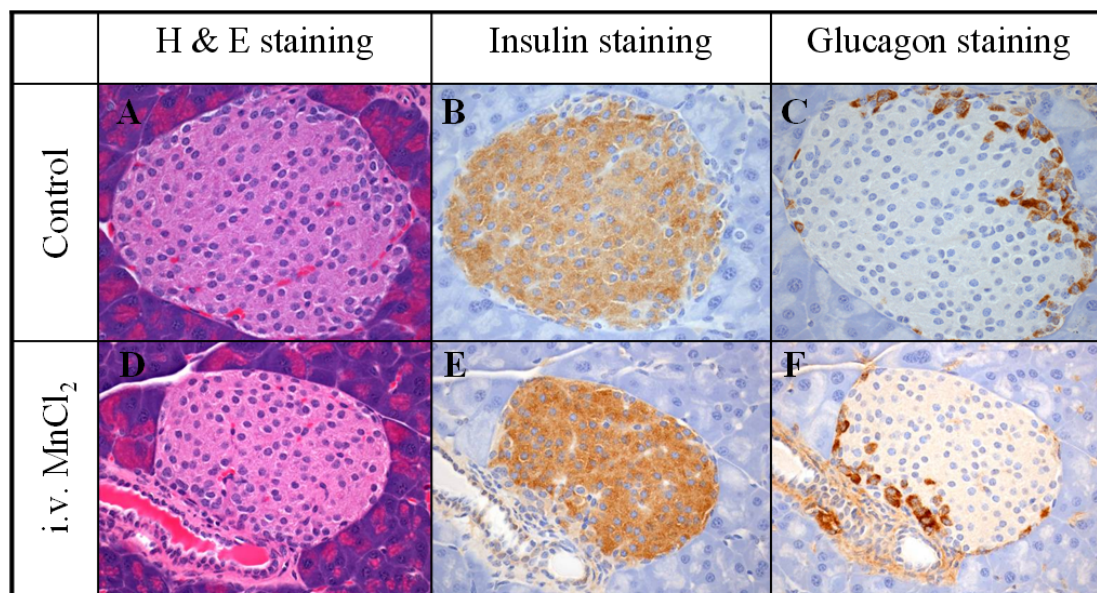


Figure 3-41. H&E stain (A, D), insulin immunostain (B, E) and glucagon immunostain (C, F) of pancreas sections in C57BL/6 mice receiving no treatment (control, A-C) and MnCl_2 infusion (2 mM, 0.1 mL; D-F). Note: H&E, insulin and glucagon stained islets represent different sections from the same islet. ($\times 40$ magnification)

3.7.2 Histological assessment of the pancreas from diabetic mice

The STZ-treated pancreas shows a reduction in islet numbers by H&E staining (Figure 3-42). In addition, STZ-treated islets were distorted with macrophage infiltration around islets (Figure 3-42). Figure 3-43 D shows pyknotic nuclei, cytoplasmic degeneration and decreased cytoplasmic eosinophilia in the STZ-treated islets compared to controls (Figure 3-43A). Insulin staining shows a decrease in the number of the β -cells (Figure 3-43 E) in the pancreas of STZ-treated mice compared to those of non-STZ-treated mice (Figure 3-43B). In contrast, there was no α -cell loss in the pancreas of STZ-treated mice compared to those of non-STZ-treated mice, as shown by glucagon staining (Figure 3-43 F v.s Figure 3-43C).

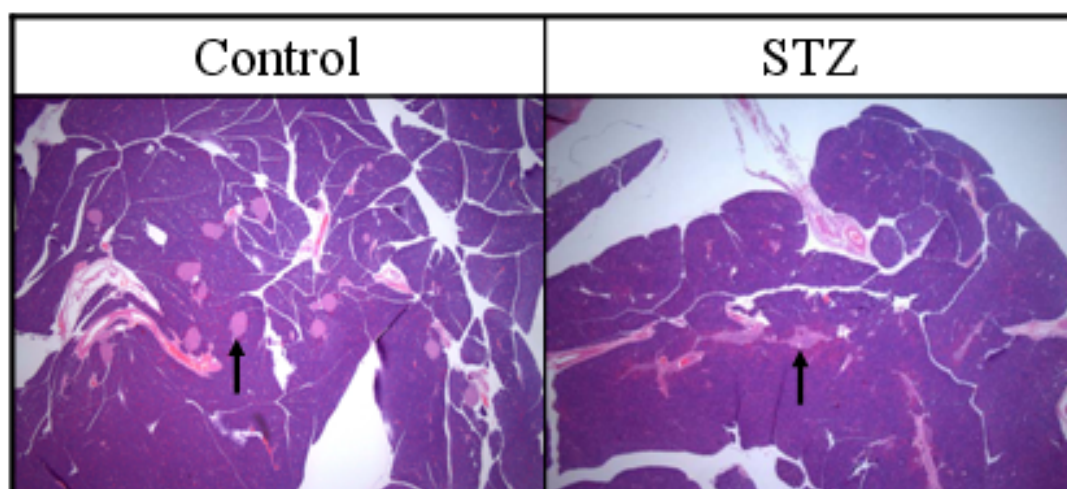


Figure 3-42. H&E staining of normal (control) and STZ-treated mouse pancreas. The islets of Langerhans (light red, arrow) are surrounded by exocrine pancreas (darker purple), scattered throughout the pancreas. ($\times 2.5$ magnification)

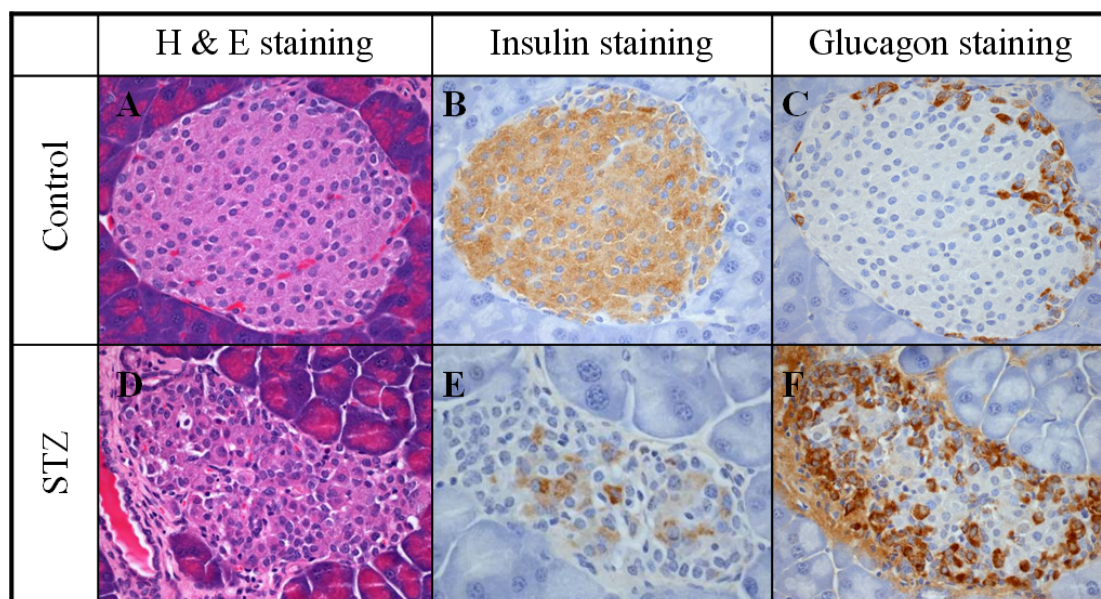


Figure 3-43. Pancreatic islet of control (A-C) and STZ-treated C57BL/6 mice stained by H&E stain (A, D), insulin immunostain (B, E) and glucagon immunostain (C, F). ($\times 40$ magnification)

4 Discussion

4.1 Gross anatomy of the mouse pancreas

The human pancreas is a solid organ located in the retroperitoneal cavity. MRI plays an important role in medical imaging of the human pancreas (Semelka and Ascher, 1993). However, imaging the mouse pancreas by MRI *in situ* is challenging as the mouse pancreas is a diffuse organ (Kara, 2005). Therefore, partial volume effect and motion artefacts can degrade the image quality of the mouse pancreas. The study of the gross anatomy is essential for the interpretation of the mouse pancreatic MR images (Grimm *et al.*, 2003). In this study, the mouse pancreas was indeed found to be a thin and diffuse organ closely adjacent to the spleen, stomach, liver, upper poles of left kidney, duodenum, jejunum and colon (Figure 3-1).

The thickest part of the mouse pancreas was found to be the gastrosplenic portion situated medial to the splenic hilum and ventral to the upper pole of the left kidney. The spleen and left kidney are solid organs and therefore the delineation of these two organs on MR images is possible. Localization of the spleen and left kidney was found to be useful in discerning the gastrosplenic portion of pancreas on MR images based in our experience.

4.2 Optimization of MRI

The SE sequence is one of the most commonly used MRI sequences. However, the pancreas in mice *in vivo* was not readily delineated in the optimized SE sequence without respiratory gating. The image quality of the mouse pancreas was poor due to significant motion artefacts from the beating heart, respiration and bowel peristalsis when respiratory gating was not employed in SE sequences. With respiratory gating, SE images are capable of delineating the gastrosplenic portion of the pancreas, although blurred boundaries for the biliary and duodenal pancreas are still produced. While employment of respiratory gating significantly improved image quality, the difficulty in maintaining a fixed respiratory rate led to effectively a range of TR values and therefore degradation in image quality. The SE sequence was limited in providing good delineation between the whole of the pancreas and surrounding tissues. Thus, it is not an ideal MRI method for imaging the mouse pancreas *in situ*.

The MP-RAGE MRI sequence is widely used for abdominal imaging (Mugler and Brookeman, 1990b) and brain morphology (Good *et al.*, 2001; van der Kouwe *et al.*, 2008). The MP-RAGE method is less prone to detrimental motion artefacts due to the nature and order of data acquisition (Lee and Price, 1994; Mugler and Brookeman, 1990b). We used a gradient echo based MP-RAGE sequence and demonstrated it to be superior to the SE sequence in delineating the mouse pancreas from adjacent organs. This is attributable to both improved contrast as well as less motion artefacts, even without respiratory gating.

Having established the superiority of the MP-RAGE sequence for imaging the mouse pancreas *in situ*, imaging parameters of the sequence including TI, flip angle and TD needed to be optimized. In order to achieve positive correlation between the changes in SI and tissue Mn^{2+} uptake, the TI value in serial MP-RAGE MRI had to be set to a value not less than the TI_{null} of the organs of interest. The signal recovery graphs show that skeletal muscle had the longest T_1 value of all the organs of interest (see Figure 3-6). The TI_{null} of skeletal muscle was approximately equivalent to 740 ms according to our study (see Figure 3-6). Therefore, the TI value in serial MP-RAGE MRI was set to 740 ms to achieve positive correlation between the changes in SI and tissue Mn^{2+} uptake.

The flip angle used is a major factor affecting SI in MP-RAGE images with lower SI at low flip angle in all the organs (see Figure 3-8). Flip angles above 25° did not increase SI, suggesting this to be the optimum value for our studies. However, even this flip angle can lead to artefacts such as blurring (Lee and Price, 1994). Previous studies have suggested a flip angle of less than $10\text{--}12^\circ$ for the MP-RAGE MRI sequence (Deichmann *et al.*, 2000; Deichmann and Haase, 1992; Jivan *et al.*, 1997) and thus, we employed a flip angle of 10° for imaging the mouse pancreas.

To measure T_1 values, a $\text{TR}_0 > 5 \times T_1$ needs to be used for MP-RAGE MRI to allow full T_1 -relaxation and prevent underestimation of R_1 values (Jivan *et al.*, 1997). However, longer TR_0 results in prolonged scan times. As a compromise between shortening scan time and allowing full relaxation of longitudinal magnetization, the TD was set to be 2000 ms. Based on the T_1 measurement results by Sahuri and Herlihy (personal communication, 2007), the T_1 values in the pancreas, kidney, liver, muscle and fat of

mouse cadavers were ~ 550 , 800 , 625 , 825 and 400 ms, respectively, using the IR-SE method. Therefore, based on their T_1 -measurements, our MP-RAGE T_1 -measurements using a TD value = 2 s gives TR_0 is $> 6 \times T_1$ for the pancreas and $> 4 \times T_1$ for skeletal muscle in the non-MnCl₂-treated mouse cadavers. In our study, there was an overestimation of T_1 -effective values by MP-RAGE. In the case, TR_0 was $\sim 4 \times T_1$ -effective for the pancreas and $\sim 2 \times T_1$ -effective for skeletal muscle in the non-MnCl₂-treated mice (Figure 3-17).

In summary, the optimized parameters for serial MP-RAGE MRI were: $TI = 740$ ms, flip angle = 10° , $TD = 2000$ ms, $FOV = 100 \times 50 \times 32$ mm (or $80 \times 40 \times 20$ mm), matrix = $256 \times 128 \times 64$ mm and 1 scan with a scan time of 4 min. With optimized parameters, MP-RAGE MRI can readily delineate the whole mouse pancreas as well as other mouse abdominal organs. Thus, the MP-RAGE sequence was chosen for imaging the mouse pancreas *in vivo* in the present study.

4.3 Validation of MP-RAGE T_1 measurement

In order to be able to measure the T_1 values of the mouse pancreas, it is necessary to use a pulse sequence that provides high resolution images with diagnostic quality. As we have shown, SE MRI is inadequate for the delineation of the mouse pancreas. MP-RAGE MRI provides excellent delineation and good contrast of abdominal organs but the relationship between SI and T_1 measurement is complex (Deichmann *et al.*, 2000; Jivan *et al.*, 1997; Kingsley, 1999; Wright *et al.*, 2008). However, the MP-RAGE T_1 measurement equation can be simplified to the standard IR equation: $SI(TI) = A - B \times e^{-TI/T_1}$ when low flip angles and the overall image-repetition time (TR_0) $> 5 T_1$ are employed (Jivan *et al.*, 1997). The calculated T_1 values by fitting the MP-RAGE data to the IR equation are named as T_1 -effective values.

In this study, T_1 was measured in three ways: IR-SE method with $TR = 15$ s; MP-RAGE method with $TD = 15$ s; and MP-RAGE method with $TD = 2$ s on samples with $MnCl_2$ concentrations of 0.04-1.00 mM, to validate the MP-RAGE T_1 measurement method. The MP-RAGE SI data were well described by the usually IR equation as well as the IR-SE data itself ($R^2 > 0.99$). These three T_1 measurement methods all show a positive linear correlation between the R_1 -effective value and $MnCl_2$ concentration ($R^2 > 0.99$) suggesting their validity for quantitative measurement *in vitro*. There was also a good agreement between T_1 measurement results by the IR-SE method with $TR = 15$ s and MP-RAGE method with $TD = 15$ s *in vitro*.

However, a difference was nevertheless observed between the T_1 measurement results by MP-RAGE method with $TD = 2$ s and IR-SE method with $TR = 15$ s. The

MP-RAGE method with $TD = 2$ s resulted in T_1 -effective measurements which deviated from the true T_1 . Deviations of T_1 -effective by MP-RAGE sequence became more significant as T_1 become longer and the T_1 -effective value tended to be an overestimate of the actual T_1 values. A $TR_0 > 5 \times T_1$ is suggested for MP-RAGE MRI since shorter TR_0 can cause an underestimation of R_1 values (Jivan *et al.*, 1997). This may explain the error of T_1 values in this experiment.

In MP-RAGE MRI, the image data are sampled during the approach to steady state and so relaxation occurs during data acquisition (Mugler and Brookeman, 1991). Large signal variation during the data sampling period can lead to artefacts such as blurring and edge enhancement (Lee and Price, 1994). Therefore, the ideal MP-RAGE MRI should be performed with a small flip angle, short TR and decreased number of phase-encoding steps to ensure identical signal amplitudes during the data sampling period (Hanicke *et al.*, 1990). In this case, MP-RAGE MRI was acquired using a smaller flip angle (10°) but a high number of phase-encoding steps in the inner loop ($n = 128$). Therefore, one possible explanation for the deviation of T_1 -effective results in phantoms would be the effect of the long readout time (1.28 s in the optimized sequence) of the MP-RAGE sequence.

3D MP-RAGE MRI can readily delineate the mouse pancreas and provide a surrogate T_1/R_1 (T_1 -effective/ R_1 -effective) measurement of $MnCl_2$ concentrations of 0.04–1.00 mM. Such concentrations are comparable to estimated tissue $[Mn]$ following $MnCl_2$ administration in our *in vivo* experiments. Therefore, this MP-RAGE method was applied to section 2.3.4-2.3.6.

4.4 Dose-dependent effects of MnCl_2

Mn^{2+} is a positive T_1 contrast agent and accumulation of Mn^{2+} in tissues can lead to an increase in SI and a shortening of T_1 value (Lin and Koretsky, 1997). In our study, MP-RAGE was demonstrated to be a semi-quantitative tool for measuring tissue [Mn] by both SI and T_1 measurements and these results were verified by ICP-AES method. A MnCl_2 dose of 2 mM, 0.1 mL infused at a rate of 0.2 mL/h was found to be the lowest dose which led to detectable signal enhancement in the optimized MP-RAGE MRI method in the mouse pancreas. Furthermore, this [MnCl_2] had no significant toxic effects. Therefore, this dose was applied for all subsequent experiments.

4.4.1 Serial MEMRI

In general, a similar signal enhancement pattern was observed in organs which readily take up Mn^{2+} , such as the pancreas, liver and kidney, following administration of MnCl_2 (2, 5, 10, 20 and 40 mM). During MnCl_2 infusion, SI rose reaching a maximum at the end of infusion period. The maximum enhancement plateau persisted for at least 96 min after the start of MnCl_2 infusion. With different doses of MnCl_2 (2, 5, 10, 20 and 40 mM), there was a dose-dependent increase in the rate of signal enhancement and steady state signal enhancement.

As can be seen, the SI plateau in the pancreas, kidney and liver lasted until the end of experiment, 96 min after the start of MnCl_2 infusion. Mn^{2+} is washed out from plasma very quick, with a plasma half life of 4.7 min but the clearance after tissue accumulation of Mn^{2+} is slow (Lin and Koretsky, 1997). Therefore, signal enhancement in organs

following plasma MnCl_2 washout is due to the uptake of Mn^{2+} which can enter cells via Ca^{2+} channels such as VGCCs (Koretsky and Silva, 2004; Silva *et al.*, 2004).

The SI plateau towards the end of the MnCl_2 infusion in most organs fluctuated within a small range. However, greater fluctuations in SI were observed in the liver, as compared to the pancreas and kidney (Figure 3-16). This may arise from the fact that the liver has a dual blood supply system. As the liver is supplied by blood from the hepatic artery and the portal vein, in theory, these can have different Mn^{2+} concentrations and thus can cause fluctuating SI from the liver. Moreover, MnCl_2 is eliminated mainly via the hepatobiliary system (Ni *et al.*, 1997) and the Mn^{2+} containing bile ducts could contribute to the SI fluctuations in the liver.

In skeletal muscle, there was a complete lack of SI change after MnCl_2 infusion at concentrations of 2-20 mM, indicating no Mn^{2+} uptake under this physiological condition. The relative constancy of the signal in the muscle makes it an ideal internal reference on time-course MEMRI. Furthermore, the volume of skeletal muscle is larger such that partial-volume effects caused by adjacent structures can be minimized. Therefore, skeletal muscle may act as an internal reference for MEMRI and this is consistent with previously published findings in which skeletal muscle was used to normalise SI in Mangafodipir-enhanced MRI (Wang *et al.*, 1997).

Following MnCl_2 infusion at a concentration of 40 mM, the SI in the skeletal muscle increased during MnCl_2 infusion and then gradually decreased. Indeed, the rate of decline in tissue Mn^{2+} was faster in skeletal muscle compared to that in the pancreas,

kidney and liver. Therefore, it is reasonable to assume that intravascular Mn^{2+} rather than intracellular Mn^{2+} contribute to the SI change in skeletal muscle following MnCl_2 infusion at a concentration of 40 mM. However, it is still unclear why there was no signal enhancement observed after Mn^{2+} administration at lower doses. VGCCs are known to be present in skeletal muscle (Catterall *et al.*, 2005; Ko *et al.*, 1997) and therefore it is also possible that Mn^{2+} can enter into skeletal muscle cells via VGCCs but may be the threshold for skeletal muscle Mn^{2+} uptake is relatively higher compared to that of other organs.

In the heart, muscle contraction is related to cellular Ca^{2+} influx through VGCCs (Wang *et al.*, 2004). Hu *et al.* has previously shown that clearance of Mn^{2+} from infarcted myocardium can be as rapid as 3-5 min after a 15 min infusion period (Hu *et al.*, 2004). Thus, MnCl_2 appears to have a very short half-life in plasma and interstitial space. Therefore, the discrepancy between the rapid plasma Mn^{2+} clearance and the extended period of steady state signal enhancement in these organs suggests that Mn^{2+} may be accumulating in the intracellular space. Based on the fact that Mn^{2+} acts as an intracellular contrast agent and the existence of dose-dependent Mn^{2+} uptake, MP-RAGE MRI may provide a semi-quantitative tool for imaging Ca^{2+} hemostasis in these tissues.

The spleen also showed low SI by MP-RAGE MRI. An increase in signal was observed in the spleen over the 30 min infusion of MnCl_2 , which was then maintained at a steady state for at least 96 min after the start of infusion. The different doses of MnCl_2 applied did show differences in signal enhancement but unlike in the other tissues, the degree of

enhancement did not correlate with the dose level (figure Figure 3-16). This may arise from the high vascularity of the spleen and the different SI of blood flowing at different rates by MP-RAGE MRI; with arterial and venous blood appearing bright and dark, respectively, (Jeffrey *et al.*, 1995). Furthermore, the SI of the slower venous blood becomes bright on administration of a T₁ contrast agent (Jeffrey *et al.*, 1995). Thus, a number of factors contribute to the resultant SI in the spleen, and explains the lack of dose-dependency.

Mn and iron have been demonstrated to be essential cofactors for many enzymes and both are transported across cell membrane via DMT1 (Roth and Garrick, 2003). Deficiency or excess of iron can influence tissue Mn levels, showing an inverse association between Mn and iron (Garcia *et al.*, 2007). It is possible that Mn exposure results in a change in tissue iron accumulation in the spleen. However, it is less likely that an acute dose of Mn²⁺ can affect iron accumulation in a short period of time such as in our experiment (< 2 h) and more studies are needed to assess the interactions between Mn and iron uptake in the spleen.

We also looked at the SI data in the heart, epididymal fat and interscapular fat (brown fat) by serial MP-RAGE MRI. However, SI data could not be measured from the same ROI throughout the 27 consecutive scans in these organs. Therefore, these data were not robust and were not included here. One possible reason for the failure to obtain SI from the same ROI may arise from motion artefacts associated with the beating heart, respiration and bowel peristalsis. Further, distension of urinary bladder was noted during the serial MEMRI which may cause the displacement of the epididymal fat.

4.4.2 T₁ measurement

There was a dose-response relationship between infused dose of MnCl₂ and shortening of T₁-effective values in the pancreas, kidney, liver and heart, and this is consistent with the results in dose-dependent serial MEMRI. In addition, this dose-dependent relationship was not observed in skeletal muscle following MnCl₂ infusion at the concentrations of 2-40 mM, and this is also consistent with the serial MEMRI results.

In the spleen, a dose-dependent relationship was not observed with serial MEMRI, a relationship was observed between the infused dose of MnCl₂ and T₁-effective measurements. The spleen has a high content of erythrocytes and lymphocytes, both of which have been shown to uptake extracellular Mn²⁺ in a dose-dependent manner (Aoki *et al.*, 2006; Matsuda *et al.*, 1989; Weed and Rothstein, 1960). The uptake of Mn²⁺ by splenic erythrocytes and lymphocytes may explain the dose-dependent effects on T₁ measurements.

Exposure to increasing doses of MnCl₂ did not affect tissue Mn²⁺ accumulation in the epididymal fat, suggesting this tissue does not uptake Mn²⁺, at least under the physiological condition in this study. This is consistent with previous *in vitro* finding showing that the T₁ values of the adipose tissue from dogs does not change after i.v. MnCl₂ (Wolf and Baum, 1983).

In the absence of MnCl₂ infusion, the T₁-effective value in the interscapular fat was significantly greater than that in the epididymal fat (Figure 3-17) which reflected their morphological differences. The latter consists of WAT whereas intercapsular fat

contains a mixture of WAT and BAT. Adipocytes of WAT contain one large fat droplet whereas those of BAT contain numerous small fat droplets, a high number of mitochondria and iron which make it brown in colour (Enerback, 2009; Hull, 1966). Unsurprisingly, WAT and BAT have different T_1 values (Houchun and Krishna, 2010) and the heterogeneity of interscapular fat may explain the difference in T_1 estimation with that of epididymal fat in our study.

Given the larger mitochondrial content of BAT and the role that Ca^{2+} plays in the function of this organelle, we expected to see an increase in Mn^{2+} . This would allow us to develop a non-invasive method to assess BAT content and function. Unfortunately, under current physiological condition (anaesthesia, immobilisation and warm temperature), no Mn^{2+} uptake was observed. Further studies are needed to determine if by stressing BAT (e.g. cold temperature, hormones, etc) may induce increased Mn^{2+} uptake.

4.4.3 Tissue [Mn] analysis by ICP-AES

ICP-AES is a multi-element analytical technique and has been used to measure the Mn content in biological samples (Jiang *et al.*, 2007). In the pancreas, kidney, liver and heart, a dose-dependent Mn^{2+} uptake was observed following a 30 min i.v. infusion of MnCl_2 at concentrations of 2-40 mM. This is consistent with previous studies showing a dose-dependent Mn^{2+} uptake in these organs in sheep (Ivan and Hidirolou, 1980; Watson *et al.*, 1973). The detection limit of Mn by our method was 0.0003 $\mu\text{g/mL}$ and the lower limit of quantification was 0.0015 $\mu\text{g/mL}$. In the muscle, spleen and adipose tissue, Mn was about or below the detection level of the technique.

It is worth noting that SI and T_1 changes measured by MRI could arise from intracellular, interstitial and intravascular Mn^{2+} accumulation whereas some of the intravascular Mn^{2+} component may not be measured by ICP-AES due to the rupture of vessels and blood loss during sample collection. This is especially relevant to the spleen due to its well known function as a blood reservoir. If the majority of Mn^{2+} was present in the intravascular space, it is reasonable that ICP-AES would only detect low tissue [Mn], failing to reflect the decreased T_1 -effective values observed in the spleen by MRI.

4.4.4 Correlation of tissue R_1 -effective with tissue [Mn]

The correlation of R_1 -effective values and tissue [Mn] were linear in the pancreas, kidney, heart, and liver after $MnCl_2$ infusion. However, tissue Mn^{2+} accumulation was tissue-variable being high in the pancreas, kidney and liver; and low in the heart. In the spleen, epididymal fat and skeletal muscle, tissue [Mn] remained low with small changes between varying $MnCl_2$ dose group. Therefore, it may be difficult to detect the small change in tissue [Mn] accumulation in these organs.

Aside from the discrepancy in the spleen, a good correlation between R_1 -effective by MP-RAGE and tissue [Mn] by ICP-AES was demonstrated in a range of tissues in our experiments. These results suggested that our serial MEMRI and T_1 -effective measurement methods can be used to estimate tissue [Mn] *in vivo* and therefore may provide possible method for assessing Ca^{2+} influx during cell activation. Ca^{2+} influx is a key step during glucose-stimulated insulin secretion in the β -cell and therefore MEMRI was used to image excited β -cells following glucose stimulation.

4.5 Tissue manganese washout

Despite its importance, there is a lack of understanding concerning Mn^{2+} transport, accumulation and clearance in live organisms following an exogenous dose (Armstrong, 2008; Pittman, 2005). While there are studies on the transport of Mn into the brain via the blood-brain barrier (Yokel, 2002), Mn^{2+} flux through non-CNS organs is relatively unknown. Mn^{2+} presents in the intravascular, interstitial and intracellular spaces after a Mn^{2+} dose. With an i.v. infusion, Mn^{2+} is rapidly cleared from the blood within minutes (Borg and Cotzias, 1958; Gerdin *et al.*, 1985) and the plasma Mn^{2+} level is restored to the baseline by 12 h in rat (Zheng *et al.*, 2000). Once leaving the plasma, Mn^{2+} distributes in tissues and then slowly washes out from tissues (Aoki *et al.*, 2006; Brurok *et al.*, 1997; Hu *et al.*, 2004; Lin and Koretsky, 1997).

In this study, a Mn^{2+} washout model was established up to 120 h after the start of MnCl_2 infusion by T_1 -effective measurement, providing useful information for future study design. Our results indicate that T_1 -effective values decreased rapidly following administration of MnCl_2 , remained at this level for over 3 h and then gradually returned to baseline, in the pancreas, kidney and liver. Combined serial MEMRI results which show a maximum enhancement plateau lasted from the end of MnCl_2 infusion for up to 60 min, suggesting a steady-state MnCl_2 accumulation period. This steady-state signal enhancement period indicates a period of constant tissue [Mn]. In skeletal muscle, T_1 -effective values remained no change over time after of MnCl_2 infusion, suggesting no obvious Mn^{2+} accumulation. Therefore, skeletal muscle may act as an internal reference in MEMRI under well controlled physiological conditions.

4.6 Glucose challenge experiment in the pancreas

The size of pancreatic β -cells is far below the spatial resolution of current MRI technology and there is also a need to use β -cell-specific contrast agent to enhance the sensitivity and specificity for imaging the functional β -cell mass (Medarova and Moore, 2009). It is known that the degree of insulin secretion is closely related to the frequency of Ca^{2+} -dependent action potentials in the pancreatic β -cell (Jacobson and Philipson, 2007). Therefore, movement of Ca^{2+} may provide a surrogate marker for β -cell function. Mn^{2+} , a surrogate for Ca^{2+} , is paramagnetic and therefore MEMRI has been used to image Ca^{2+} -related cellular activation in the neuron (Silva *et al.*, 2004) and heart (Hu *et al.*, 2001).

Mn^{2+} accumulates in high concentrations within the pancreas following an exogenous dose (Ni *et al.*, 1997). It has been shown to inhibit insulin secretion at very high Mn^{2+} concentrations following glucose stimulation *in vitro* (Rorsman *et al.*, 1982). Therefore, a balance between minimizing potential toxicity arising from MnCl_2 and the need for high tissue contrast for localization of the mouse pancreas is vital. The dosage we have implemented in our MEMRI protocol is significantly lower than the reported LD_{50} for i.v. MnCl_2 in mice (Silva *et al.*, 2004). Crucially, we have shown that MnCl_2 infusion (2 mM, 0.1 mL) has no effects on blood glucoses levels by i.p. injection of glucose (2 g/kg body weight). Further, MnCl_2 infusion at this dose did not affect the islet and β -cell numbers by histological analysis including H&E and immunohistochemistry staining.

For imaging the pancreatic β -cell activation, both SI profiles and T_1 measurements were obtained in healthy mice with and without glucose challenge. SI profiles of a STZ-treated diabetic mouse model were also obtained for comparison. Two articles highly related to this project were published during the course of this study, one *in vitro* and one *in vivo* experiment. The *in vitro* study showed that β -cells take up Mn^{2+} in response to glucose stimulation in the presence of $MnCl_2$ and the increased cellular Mn^{2+} uptake yielded an SI increase of 200 % by MRI (Gimi *et al.*, 2006). The *in vivo* study showed that MEMRI can detect a 51 % increase in the maximal SI of the pancreas after glucose stimulation, as compared to control. The results from our study are discussed below in light of these papers.

4.6.1 Healthy mice

This *in vivo* MRI protocol requires that I select a mean SI value from an ROI from the pancreas, as MEMRI currently lacks the resolution to study single β -cells or even islets. Despite the fact that the endocrine pancreas contributes only 1-2 % of the total pancreatic volume, I have demonstrated *in vivo* a statistically greater SI in the pancreas following a glucose challenge compared to the control group. The serial MEMRI protocol provided a sufficient temporal resolution (4 min) to detect a significant difference in SI, starting at 4 min post i.p. glucose injection. I have recorded a peak in circulating glucose at around 30 min post i.p. injection, similar to that observed in previous C57BL/6 mice studies (Sone and Kagawa, 2005). The timing of this peak in blood glucose also corresponds to a peak in temporal SI, suggesting that the MEMRI data may accurately reflect pancreatic insulin secretion. Therefore, MEMRI appears to provide a quantitative representation of β -cell activity.

Thus, I have demonstrated that MEMRI is an effective means of non-invasively monitoring β -cell function, in agreement with a recent publication which showed a 50 % increase in normalized pancreas SI after glucose stimulation (Antkowiak *et al.*, 2008). In their study, C57BL/6 mice (~ 29 g) were fasted for 2 h prior to MRI and treated with i.p. MnCl_2 ($0.1 \mu\text{mol/g}$) and i.v. glucose (1.5 g/kg). The MnCl_2 dose they used was $\sim 20 \text{ mg/kg}$, which is half the dose of LD_{50} and 10 times more than the dose in my study. It is clear that the MnCl_2 dose they used can yield greater SI increases but the dose I used is less toxic, with minimal effect on β -cell function. They used a 2D MP-RAGE without physiological gating to image the mouse pancreas. In agreement with their study, I found that a 3D MP-RAGE sequence without gating can better delineate the mouse pancreas *in vivo*. Flip angle is always a concern (see section 4.2) when performing MP-RAGE sequence and a flip angle of less than $10\text{-}12^\circ$ for the MP-RAGE MRI sequence has been suggested (Deichmann *et al.*, 2000; Deichmann and Haase, 1992; Jivan *et al.*, 1997). A flip angle of 20° was used in their study, whereas I used a flip angle of 10° . Clearly, a high flip angle achieves greater SI difference in the pancreas following MnCl_2 dose but with the penalty of poor image quality (Holsinger A and Riederer SJ, 1990).

In the study by Antkowiak and colleagues, a $\text{TI} = 580 \text{ ms}$ was used. I measured TI_{null} of the pancreas to be 500 ms (Figure 3-6) and therefore, a $\text{TI} = 580 \text{ ms}$ ($> \text{TI}_{\text{null}}$ of the pancreas) yields positive correlation between changes in SI and tissue $[\text{Mn}^{2+}]$ accumulation. However, their choice of TI value renders it difficult to analyse the SI changes in other insulin target organs, such as the liver and skeletal muscle. TI_{null} values for the liver and skeletal muscle were 600 ms and 740 ms , respectively and therefore, using a TI of 580 ms means that the SI change is not directly correlated to tissue $[\text{Mn}^{2+}]$.

Furthermore, SI data in the pancreas was normalized to the SI in the liver to account for variations in the Mn^{2+} delivery, whereas I normalized the pancreatic SI at each time point to the baseline pancreatic SI prior to Mn^{2+} infusion. Insulin-target organs are not ideal internal standards, as tissue [Mn] may change following glucose stimulation. Furthermore, the liver is the main organ for Mn^{2+} excretion and a target organ for glucose homeostasis (Barritt *et al.*, 2008) and the selection of liver as an internal standard further complicates the situation. Thus, I do not recommend that the liver SI should be used to normalise the pancreas SI.

Although Antkowiak *et al.* (2009) showed that MEMRI can detect a significant increase in the maximal SI of the pancreas after glucose stimulation; we have further validated the application of MEMRI by obtaining T_1 measurements to confirm Mn accumulation in tissues. Also, I employed non-MRI methods to understand the changes in the MEMRI enhancement profile by analysing tissue [Mn] *in vitro* by ICP-AES, LA-ICP-MS and XRF.

T_1 -effective/ R_1 -effective measurements did not suggest an increase in Mn^{2+} accumulation in the mouse pancreas following glucose stimulation, despite the serial MEMRI findings. The T_1 -effective measurements took ~ 22 min whereas the temporal resolution for the serial MEMRI experiment is ~ 4 min. Relatively poor spatial resolution may explain the inability of T_1 -effective/ R_1 -effective measurements to detect the increase in Mn^{2+} .

Bulk tissue Mn measurements by ICP-AES also failed to detect differences between the pancreas with and without glucose stimulation. Errors may occur due to detection limits and sample contamination. Pancreatic islets consists of only 1-2 % of pancreatic mass and thus tissue Mn mapping with high spatial resolution at the level of the islet is needed to visualize the Mn distribution inside and outside the islet or β -cell. ICP-AES is a bulk analytical technique and therefore LA-ICP-MS was also employed for a 2D elemental mapping.

A pilot study of Mn and iron mapping of frozen sections of mice pancreata was performed at a resolution of 100 μ m with and without glucose activation by LA-ICP-MS. H&E staining of adjacent slice was performed to localize the pancreas. The Mn map shows a general higher level of Mn in the glucose-stimulated pancreas compared to that of vehicle-treated pancreas (Figure 3-24). However, both pancreata samples also contained spleen which had different iron levels suggesting that SI is not comparable between samples as iron concentrations should be comparable between the spleens. Thus, direct comparison of elemental concentrations is allowed within each sample but not across samples and our LA-ICP-MS method requires further refinement and calibration for generation of quantitative elemental maps. Despite this, our LA-ICP-MS data does demonstrate Mn to be lower in the spleen than in the pancreas, consistent with the serial MEMRI and T_1 -effective measurement data (see section 3.4). Further, the LA-ICP-MS data showed higher iron concentrations in the spleen than in the pancreas as expected from the known function of the spleen to act as a reservoir for erythrocytes (Hamza and Kaufman, 2009).

A pilot study of Mn and Zn mapping for the paraffin wax sections of the pancreas was also performed in mice with and without glucose activation by XRF. Zn mapping and H&E staining of adjacent slice were used to localize the islet. A high degree of correlation was observed between Mn and Zn distribution in the glucose-stimulated pancreas whereas a lesser degree of correlation was observed in the control pancreas. These findings suggest specific Mn accumulation in areas of high Zn, i.e., within or at least in the vicinity of β -cells. The lack of exact correspondence between Mn mapping and islet distribution may be due to the fact that not all β -cells are activated following a glucose load. Since XRF was performed a resolution of 300 μm , partial volume effects may contribute to the lack of correspondence. The mean normalized Mn intensity was higher in the glucose-treated pancreas than the control by XRF. Thus, the employment of metal mapping by XRF suggests that the increase in signal enhancement by MEMRI in the activated pancreas may indeed result from entry and accumulation of Mn along with Ca^{2+} during glucose-mediated β -cell activation.

4.6.2 Diabetic mouse model

STZ is diabetogenic and its structure is similar to glucose and can be taken up by β -cells via GLUT-2, resulting in specific β -cell toxicity (Wang and Gleichmann, 1998). Histological assessments of pancreatic islets in STZ-treated diabetic mice illustrated β -cell degeneration and loss and macrophage infiltration with α -cell retention, therefore making this a good type 1 diabetic mouse model.

Glucose stimulation resulted in a significant increase in pancreatic SI by MEMRI in healthy mice but not in STZ-induced diabetic mice. Since STZ treatment induces β -cell necrosis but does not appear to affect non- β -cells, it is very likely that β -cells are responsible for the further Mn^{2+} uptake following glucose stimulation in the pancreas of Mn^{2+} -treated animals. Therefore, MEMRI appears to provide a quantitative representation of β -cell loss.

4.7 Glucose challenge in insulin-target organs

4.7.1 Skeletal muscle

In this study, *in vivo* MEMRI was also carried out to assess glucose uptake activity in skeletal muscle in healthy mice and the diabetic mouse model. An increase in SI following glucose stimulation was detected in healthy mice. A glucose load can induce an increase in plasma insulin levels in anesthetized animals and the resultant hyperinsulinemia state initiates the muscle glucose uptake pathway. VGCCs are known to be present in skeletal muscle (Catterall *et al.*, 2005; Ko *et al.*, 1997) and therefore Mn^{2+} may enter into skeletal muscle cells through Ca^{2+} transport pathway upon glucose stimulation in the presence of extracellular Mn^{2+} .

In skeletal muscle, glucose homeostasis is regulated by insulin-mediated and contracture-stimulated glucose uptake (Goodyear and Kahn, 1998). Exercise-mediated skeletal muscle glucose uptake is related to an increase in intracellular Ca^{2+} but the increase is due to release of Ca^{2+} from intracellular stores not cellular Ca^{2+} influx (Goodyear and Kahn, 1998; Rose and Richter, 2005). The role of Ca^{2+} in insulin-mediated glucose uptake is still unclear (Lanner *et al.*, 2008). However, as mice used in our studies were maintained under anaesthesia throughout the MRI scan, the muscle was in a resting state. Thus, exercise-induced skeletal muscle uptake is unlikely to be responsible for the increase in SI in our MEMRI. It is therefore reasonable to suggest that the SI increase in skeletal muscle following glucose stimulation is due to insulin-mediated skeletal muscle glucose uptake.

Cellular Ca^{2+} influx has been shown to be an important modulator of insulin-mediated skeletal muscle glucose uptake *in vitro* (Lanner *et al.*, 2006). In agreement with their result, we demonstrate an increase of Mn^{2+} influx into skeletal muscle following a glucose load in healthy mice. Therefore, MEMRI may be used as a non-invasive tool to detect the insulin-mediated glucose uptake in skeletal muscle.

Impaired insulin-mediated skeletal muscle glucose uptake is present in both type 1 and type 2 diabetes (Napoli *et al.*, 1995). Increase in SI change was expected in glucose-stimulated skeletal muscle in STZ-treated diabetic mice but of a smaller magnitude compared to that in healthy mice. However, our results show that glucose induces Mn^{2+} influx, was of similar magnitudes to those of healthy mice. While it is reasonable to conclude that the Mn^{2+} influx is associated with muscle glucose uptake in healthy subjects, the similar increase in skeletal muscle of STZ mice is more difficult to explain. Antioxidant enzymes such as MnSOD play a role in insulin sensitivity in skeletal muscle (Hoehn *et al.*, 2009), and the administration of Mn may increase muscle insulin sensitivity in the STZ mice so as to compensate for the insulin deficiency as a result of STZ destruction of β cells. Further investigations involving assessments of muscle insulin sensitivity and measurements of blood insulin are required to determine whether Mn administration can restore insulin sensitivity to a significant extent in STZ mice.

4.7.2 Liver

In the liver, Ca^{2+} is involved in a wide range of cellular activities, as discussed in section 1.5.2. Further, increased SI in the liver following Mn^{2+} infusion can arise from

its accumulation in bile ducts, vessels, interstitial spaces as well as in hepatocytes. These SI changes reported here may not be a simple reflection of increased hepatocellular Mn^{2+} uptake as a result of Ca^{2+} -mediated processes. Mn^{2+} uptake was high by the non-stimulated liver as shown in Figure 3-18 and further cellular Mn^{2+} entry following stimulation may be masked by the already high tissue Mn^{2+} accumulation. Therefore, it is a challenge to interpret the MEMRI data in the liver. As expected, there was no difference in Mn^{2+} accumulation between glucose-stimulated or control groups by both MEMRI and ICP-AES data in healthy and diabetic mice. This is likely to be the case as the SI increase in the liver is as high as 120 % and therefore, the large influx of Mn^{2+} into this region may mask the relatively smaller cellular Mn^{2+} influx.

4.7.3 Adipose tissue

It is reasonable to hypothesize that MEMRI could be used to image the function of the adipose tissue in glucose homeostasis. Serial MEMRI was therefore performed in these tissues. Unfortunately, the ROIs from the epididymal fat had to be moved for each scan to compensate for motion throughout the experiment, as discussed in section 4.4.1. Furthermore, significant difference in SI was found between glucose-stimulated epididymal fat and control prior to the application of i.p. stimuli when both groups were treated with only MnCl_2 infusion. These findings suggested that our current MEMRI protocol, designed for the pancreas, may not be readily applied to measure the SI change in the epididymal fat. Clearly, future work is needed to further optimize the serial MEMRI protocol for imaging the epididymal fat. No difference was observed in T_1 -effective values of the epididymal fat in mice receiving glucose or vehicle after MnCl_2 infusion. This may be due to the lower tissue $[\text{Mn}]$ in the epididymal fat, consistent

with the ICP-AES results which showed that some of the tissue [Mn] in the epididymal fat was lower than the detection limit.

The serial MEMRI results for the interscapular fat were also unreliable as again a constant ROIs could not be obtained, probably due to effects arising from motion associated with heat from beating heart, respiration and bowel peristalsis. In the interscapular fat, there was no difference in SI in mice with and without stimulation following MnCl_2 infusion in the serial MEMRI study. Moreover, margins of the interscapular fat can only be delineated from 8 out of 12 animals in the study. The MP-RAGE sequence employed here was optimized for the pancreas and therefore, may not be ideal for imaging the interscapular fat. T_1 measurement study was not performed in the interscapular fat as a lack of dose-dependent relationship between infused Mn^{2+} dose and changes in T_1 values as described in section 3.4.2.

4.8 Limitations and future works

While MEMRI allowed the *in vivo* imaging of pancreatic β -cell activation, there are limitations to the method presented here. Firstly, delineation of the mouse pancreas by MP-RAGE MRI is difficult, and the inter-observer variability for the selection of ROIs for the mouse pancreas had not been assessed in this study due to a lack of experienced operators. Secondly, the Mn^{2+} -based contrast agent used in this work, MnCl_2 , is toxic and may effect β -cell function. Thirdly, this work did not show dose-dependent glucose stimulation of the mouse pancreas by MEMRI. Finally, the effect of MnCl_2 on β -cell insulin secretion was not tested in these studies.

Future work would include the use of a FDA-approved Mn^{2+} -based contrast agent, MnDPDP , to replace MnCl_2 which may have toxic effects. Other studies include the employment of MEMRI to quantitatively assess β -cell function over a range of glucose doses. An *ex vivo* study can be performed to monitor whether β -cells following glucose stimulation demonstrate enhancement compared to non-stimulated β -cells in the presence of extracellular Mn^{2+} by T_1 -weighted MRI, further supporting the methodology in the *in vivo* studies presented here.

4.9 Conclusions

Exocytosis of insulin granules in pancreatic β -cells is regulated by cellular influx of extracellular calcium ions (Ca^{2+}). Using Mn^{2+} as a surrogate for Ca^{2+} , I have demonstrated that serial MEMRI can be used to assess β -cell function following glucose stimulation in normal and diabetic mice *in vivo* and may offer a valuable diagnostic tool for therapy against diabetes. I have also demonstrated that serial MEMRI can detect an increase in tissue Mn^{2+} accumulation in skeletal muscle following a glucose load, indicating an increase in cellular Ca^{2+} influx during skeletal muscle glucose uptake. Therefore, my MEMRI methodology may be a powerful tool to assess glucose homeostatic processes *in vivo* and can improve our understanding of the mechanisms underlying the development of diabetes. The relationship between the number of activated β -cells, the degree of β -cell activation and Mn^{2+} entry remain unclear. Moreover, glucose administration has been shown to increase islet vascularity (Jansson, 1988; Jansson and Hellerstrom, 1983) which might contribute to the tissue Mn^{2+} uptake. So while VGCCs are an obvious candidate for the increased Mn^{2+} entry into the pancreas following stimulation with glucose, it is important to further clarify the origin of signal enhancement observed by MEMRI.

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Publications

NMR in Biomedicine (In press)

Manganese Enhancement in Non-CNS Organs

Li-Wen Lee^{1,2}, Po-Wah So³, Anthony N. Price¹, James RC Parkinson¹, David J Larkman¹, Jane Halliday⁴, Simon M. Poucher⁴, John A. T. Pugh⁵, Alan G. Cox⁵, Cameron W. McLeod⁵, Jimmy D. Bell^{1*}

¹Metabolic and Molecular Imaging Group, MRC Clinical Sciences Centre, Hammersmith Hospital Campus, Imperial College London, London, UK; ²Department of Diagnostic Radiology, Chang Gung Memorial Hospital-Chiayi, Chang Gung University College of Medicine, Chiayi, Taiwan; ³Preclinical Imaging Unit, King's College London, Institute of Psychiatry, James Black Centre, Denmark Hill Campus, London, UK; ⁴Translational Sciences and Cardiovascular and Gastrointestinal Discovery Department, AstraZeneca Pharmaceuticals, Macclesfield, UK; ⁵Centre for Analytical Sciences, University of Sheffield, Sheffield, UK

Key words: MEMRI, manganese, pancreas, non CNS organs, MP-RAGE

Abbreviations: GEE, generalized estimating equations; ICP-AES, inductively coupled plasma atomic emission spectrometry; IR-SE, inversion recovery spin echo; MEMRI, manganese-enhanced magnetic resonance imaging; MP-RAGE, magnetization-prepared rapid gradient echo; ROI, region of interest; SI, signal intensity; VGCC, voltage-gated Ca²⁺ channels.

Abstract

Manganese-enhanced magnetic resonance imaging (MEMRI) is a novel imaging technique capable of monitoring calcium influx, *in vivo*. Manganese (Mn²⁺) ions, similar to calcium ions (Ca²⁺), are taken up by activated cells where their paramagnetic properties afford signal enhancement in T₁-weighted MRI methodologies. In this study we have assessed Mn²⁺ distribution in mice using magnetization-prepared rapid gradient echo (MP-RAGE) based MRI, by measuring changes in T₁-effective relaxation times (T₁-eff), effective R₁-relaxation rates (R₁-eff) and signal intensity (SI) profiles over time. The manganese concentration in the tissue was also determined using inductively coupled plasma atomic emission spectrometry (ICP-AES). Our results show a strong positive correlation between infused dose of MnCl₂ and the tissue manganese concentration. Furthermore, we demonstrate a linear relationship between R₁-eff and tissue manganese concentration and tissue-specific Mn²⁺ distribution in murine tissues following dose-dependent Mn²⁺ administration. This data provides an optimized MnCl₂ dose regimen for an MP-RAGE based sequence protocol for specific target organs and presents a potential 3D MRI technique for *in vivo* imaging of Ca²⁺ entry during Ca²⁺-dependent processes in a wide range of tissues.

Introduction

Manganese-enhanced magnetic resonance imaging (MEMRI) is an emerging contrast agent based MRI technique that can be used for high-resolution anatomical and functional imaging *in vivo* (Kuo et al., 2007; Lin and Koretsky, 1997; Silva et al., 2004). The technique is based upon the ability of paramagnetic manganese ions (Mn²⁺) to alter the proton MRI signal, resulting in an increase in signal intensity (SI) by T₁-weighted MRI (Fornasiero et al., 1987; Mendonca-Dias et al., 1983). The change in SI is proportional to the concentration of Mn²⁺ within a given region of interest (ROI) and yields a quantitative measure of the transport and accumulation of Mn²⁺ in specific tissues (Lee and Koretsky, 2004; Mendonca-Dias et al., 1983). Importantly, systemic administration of MnCl₂ results in an increased SI from both cells and extracellular

components including blood (Kang et al., 1984) and after wash out of MnCl₂ from blood, SI only arises from intracellular Mn²⁺ (Bremerich et al., 2000). Hu *et al.*, have shown that Mn²⁺ can wash out of the infarcted myocardium as rapid as 3-5 min after MnCl₂ infusion (Hu et al., 2004). Unlike other T₁ contrast agents such as gadolinium chelates which remain extracellular, Mn²⁺ offers a unique perspective of tissue, mapping SI according to cellular function, i.e. it is an activity-dependent contrast agent.

In addition to its paramagnetic properties, Mn²⁺ has an ionic radius comparable to the calcium ion (Ca²⁺) (Shannon, 1976) and behaves similarly in biological systems, entering and exiting various tissues via Ca²⁺ transport systems (Cross et al., 2007; Narita et al., 1990; Rorsman et al., 1982; Simpson et al., 1995; Wollheim and Sharp, 1981). In excitable cells, voltage-gated Ca²⁺ channels (VGCC) in the cell membrane open following stimulation, resulting in enhanced uptake of Mn²⁺ (Drapeau and Nachshen, 1984). As the change in SI is proportional to the concentration of Mn²⁺, the resulting contrast enhancement can therefore be used as a direct marker for Ca²⁺-dependent cellular activity and function. Based upon these principles, MEMRI has been used to image cellular activity in the brain and heart (Morita et al., 2002; So et al., 2007) and also trace neuronal tracts in visual and olfactory pathways (Silva and Bock, 2008; Silva et al., 2004).

The kinetics of Mn²⁺ transit in the brain have been established using both MRI and non-MRI methodologies (Kuo et al., 2007; Silva and Bock, 2008; Teeguarden et al., 2007). There are differences in Mn²⁺ uptake in different organs with a high degree of Mn²⁺ accumulation in the pancreas, kidney and liver; a moderate uptake in the brain and spleen; and low levels in adipose tissue and skeletal muscle, regardless of the exogenous dose (Chauncey et al., 1977; Sotogaku et al., 2000; Spiller et al., 1988; Takeda et al., 1998). However, despite sufficient levels of manganese reaching non-brain tissues, imaging these regions is often technically demanding with motion artifacts arising from the heart, respiration and bowel peristalsis, degrading the image quality and producing suboptimal diagnostic accuracy (McRobbie, 2007). To overcome these technical challenges magnetization-prepared rapid gradient echo (MP-RAGE) sequences are particularly well suited; providing detailed anatomical structures with high tissue contrast and short acquisition times (McRobbie, 2007; Mugler and Brookeman, 1991). MP-RAGE is less prone to detrimental motion artifacts in comparison with spin echo methods due to the nature and order of data acquisition relative to the timing of various motions such as peristalsis and respiration (Mugler and Brookeman, 1990b).

In this study, we determined the time-course effects of dose-dependent Mn^{2+} administration in non-CNS organs to aid implementation of MEMRI-based techniques for imaging non-CNS Ca^{2+} -mediated cellular activity. We developed and applied *in vivo* 3D MP-RAGE MRI, obtaining SI profiles prior to, during and after intravenous (i.v.) infusion of manganese chloride ($MnCl_2$). Conventionally, T_1 is measured by spin echo-inversion recovery (IR-SE) but often such data is susceptible to motion artifacts when abdominal organs are imaged (see above), and hence our use of MP-RAGE MRI in these studies. Although the calculation of T_1 from MP-RAGE-based methods can be complex (Deichmann et al., 2000; Jivan et al., 1997; Wright et al., 2008) the equation relating SI to T_1 at varying inversion times (TI) can be approximated by the IR equation when low flip angles are used and the overall image repetition time (TR_0) is large relative to T_1 (Jivan et al., 1997). We have employed a similar method to assess T_1 values, which we have termed T_1 -effective (T_1 -eff), in recognition of the approximations made when using the standard IR equation to calculate T_1 values from MP-RAGE data. Correlation between MRI data and absolute tissue manganese (Mn) content is currently only available in the brain (Chuang et al., 2008) and heart (Waghorn et al., 2008), and even less is known about the systemic distribution of Mn^{2+} *in vivo*. Here we record T_1 -eff measurements in the plateau phase of the SI profile following Mn^{2+} administration and correlate this data with absolute tissue Mn, obtained by inductively coupled plasma atomic emission spectrometry (ICP-AES).

Materials and Methods

Sample (Dosing solution) Preparation

A stock solution of manganese chloride tetrahydrate (1M $MnCl_2 \cdot 4H_2O$, Sigma-Aldrich Co, UK) was diluted with distilled water to concentrations ranging from 0.04-40mM.

Animal Preparation and Treatments

All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986. Adult male C57BL/6 mice (Harlan, UK) were housed in individually ventilated cages and maintained on a 12:12 hour light-dark cycle (light on at 7am and off at 7pm). Animals of similar body weights were used within experiments to minimize inter-group variation. Animals were allowed *ad libitum* access to food and water. Prior to scanning, mice were fasted overnight from 5pm but allowed *ad libitum* access to water. All MRI scans were performed in the early light cycle of the day. Mice were weighed prior to scanning and then anaesthesia induced using a mixture of 4-5 % isoflurane, 2 L/min oxygen. Anaesthesia was maintained with 1.5-2 % isoflurane, 1 L/min oxygen via a nose cone. During scanning, respiration and rectal temperature were monitored continuously. Animal core temperature was maintained by a warm air heating system (SA instruments, NY, USA) with automated feedback control system at a set point of 37°C. As $MnCl_2$ infusion causes an initial stimulation followed by depression of respiration (more pronounced at higher Mn^{2+} concentrations) (Silva et al., 2004), anaesthetic levels were adjusted during the imaging to maintain steady state respiration.

Experiment 1: Measurement of *in vitro* T_1 (and R_1) values by IR-SE and MP-RAGE

To compare T_1 and T_1 -eff values IR-SE and MP-RAGE were performed on 14 different concentrations of manganese (0.04-1.00mM), covering the range of T_1 values in the subsequent *in vivo* study (experiment 2). Tubes containing 14 increasing concentrations of $MnCl_2$ were

positioned in a sample holder prior to scanning. The samples were placed within a quadrature volume coil (35mm internal diameter, Magnetic Resonance Laboratories, Oxford, UK) on a 4.7T VMRI scanner (Varian Inc., Palo Alto, USA). T_1 measurements were first made using IR-SE with the following parameters: TI = 20, 40, 60, 80, 100, 140, 200, 300, 400, 500, 700, 900, 1200, 1500, 2000, 2500, 3000 and 4500ms; TR (repetition time) = 15s; TE (echo time) = 11.97ms; matrix size = 256 x 128; FOV (field of view) = 90 x 45mm; number of repetitions = 2; and a single slice, 2mm thick.

T_1 is typically measured by spin echo-inversion recovery (IR-SE) but such data is susceptible to motion artifacts when abdominal organs are imaged. We have overcome these issues by using an MP-RAGE sequence. The equation relating SI to T_1 at varying inversion times (TI) can be approximated by the IR equation when a low flip angle is used ($< 8^\circ$) and the overall image repetition time (TR_0) is large relative to T_1 (greater than 5 times the T_1) (Jivan et al., 1997). We have employed a similar method to assess T_1 values which we have termed " T_1 -effective" (T_1 -eff) in acknowledgment of the limitations of the rapid imaging approach. A 3D MP-RAGE sequence was used to measure T_1 -eff using the following parameters: TI = 100, 200, 500, 1000 and 2400ms; TR = 10ms; TE = 2.4ms; TD = 2s; matrix size = 256 x 128 x 64; FOV = 80 x 40 x 20mm; number of repetitions = 1 and a flip angle = 10° . (From previous unpublished data, tissue T_1 values are in the range 500-1000ms; 550ms and 800ms from pancreata and skeletal muscle, respectively, in dead mice using IR-SE). Overall image repetition time (TR_0) = TD + TI + (phase encoding steps x TR), therefore $TR_0 = 2000ms + TI + (128 \times 10ms)$ and TR_0 is therefore $> 3380ms$. A long TR_0 was chosen to allow sufficient magnetization recovery, but low enough to keep the overall scan time manageable. T_1 and T_1 -eff values were calculated from the IR-SE and MP-RAGE MRI data obtained from the various $MnCl_2$ solutions, respectively, using the following equation: $SI_{TI} = M + B \times (\exp(-TI/T_1))$, where SI_{TI} is the SI at inversion time TI, and M is the magnetization at time $t = 0$. R_1 (and R_1 -eff) values were calculated by taking the reciprocal value of T_1 (and T_1 -eff). To compare R_1 and R_1 -eff, the percentage estimation error was calculated as follows: percentage estimation error = $(R_1\text{-eff} - R_1) / R_1 \times 100 \%$.

Experiment 2: Correlation of MP-RAGE and ICP-AES data following Mn^{2+} infusion

Male C57BL/6 mice ($n = 15$, $22.1 \pm 0.2g$ after overnight fasting) were prepared as above. Animals were divided into 5 groups of 3 animals and given different doses of $MnCl_2$. There were no significant differences in body weight between the groups (data not shown). Prior to MRI, a 27 gauge butterfly cannulae was inserted i.v. into the tail vein for a 30min infusion of aqueous $MnCl_2$ solutions (2, 5, 10, 20 or 40mM) by a syringe pump (0.2ml/h, 0.1ml final volume). The highest dose of $MnCl_2$ employed (40mM) is the LD_{50} for C57BL/6 mice (Silva et al., 2004). A further 6 animals ($21.7 \pm 0.4g$ after overnight fasting) were also imaged without $MnCl_2$ infusion. Employing such high $MnCl_2$ doses provides greater signal in MEMRI experiments, however, due to possible toxicity, administration of as low a dose as possible (2mM) is preferred, especially when considering its use in longitudinal experiments.

3D MP-RAGE MRI

MRI scans were performed using a 4.7T VMRI scanner at 6min after the cessation of Mn^{2+} infusion. MP-RAGE parameters employed were as in experiment 1, with a total scan time of 22.5min. SI of regions of interest (ROIs, see below) at different TI(s) were measured and T_1 -eff values

calculated. After the T_1 -eff measurements, animals were euthanized and tissues: pancreas, spleen, kidney, liver, fat, skeletal muscle and heart collected (corresponding to 36min after $MnCl_2$ infusion). Tissues were frozen on dry ice and kept at $-80^\circ C$ prior to assessment of Mn by ICP-AES (see below). Of the 6 control mice that did not receive an infusion of $MnCl_2$, three of them were euthanized following MRI scanning (22.5min) and organs collected for ICP-AES analysis. T_1 -eff values were calculated as in experiment 1.

ICP-AES Analysis

Frozen tissue samples were weighed, and dried ($110^\circ C$, 48h) to a constant weight, prior to acid digestion. Samples were digested using a mixture of concentrated Aristar nitric acid (3ml) and concentrated Aristar perchloric acid (0.5ml) in a heating block, initially at $150^\circ C$ then progressively ramped up to a temperature of $200^\circ C$ over a period of 2h. The resultant clear solutions were quantitatively transferred to a 15ml graduated plastic tube and made up to a volume of 10ml with 1 % nitric acid. The resultant solution was then analyzed using a SPECTRO VISION (CIROS) ICP spectrometer. Data is presented as $\mu g/g$ dry mass.

Experiment 3: Dose-dependent tissue SI time-course following $MnCl_2$ infusion

Male C57BL/6 mice were fasted overnight ($n = 15$, $19.7 \pm 0.4g$ after overnight fasting) and prepared as detailed above. In order to obtain a dose-dependent, time-course profile of Mn^{2+} entry into various tissues, different doses of $MnCl_2$ (2, 5, 10, 20 or 40mM, 0.2ml/h, 30min, final volume = 0.1ml, $n = 3$ /group) were infused i.v. via the tail vein (as in experiment 2). MP-RAGE scans were performed, with the same parameters as in experiment 1, except $TI = 740ms$, $FOV = 100 \times 50 \times 32mm$ with a total acquisition time of 4min. The TI value of 740 ms was chosen to be as close to the TI_{null} of unenhanced tissues such that the sequence provides heavily weighted images so as to be sensitive to manganese uptake. Three consecutive datasets were collected prior to Mn^{2+} infusion to provide baseline data. The tail vein Mn^{2+} infusion was then started and a series of 24 consecutive datasets collected: data was collected during the 30min $MnCl_2$ infusion (0.2ml/h, 0.1ml) and then for a further ~66min after the end of infusion. The time-course data is presented as the % change in SI from baseline: % SI change = $(SI_t - SI_0) / SI_0 \times 100 \%$. SI_0 is the average SI of three MRI datasets acquired before the start of $MnCl_2$ infusion and SI_t the SI at time t .

The choice of flip angle value was based on preliminary experiments. A C57BL/6 mouse was scanned as detailed above without administration of $MnCl_2$ and the value of the flip angle for the MP-RAGE sequence varied: 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50° .

Image analysis

MRI images were analysed using Image J software (Image J 1.3.1, NIH, USA). SI from ROI was selected from each tube in the *in vitro* experiments. For *in vivo* tissue analysis, SI readings were obtained from ROIs drawn in the gastrosplenic portion of pancreas, left kidney cortex, liver, left thigh (skeletal muscle), spleen, epididymal fat and heart aided by reference to a standard mouse atlas (Iwaki and Hayakawa, 2005). ROIs (Supplementary Figure 2) were selected centrally within the organs to minimize partial volume effects, and the same ROIs used for images collected at the different TI values and at different time points. It was possible to apply the same ROIs in the

pancreas, kidney, liver, heart, spleen and muscle for all 5 acquisitions for T_1 -eff measurement in experiment 2 due to the lack of motion artefacts in the image. Likewise, the same ROIs could be applied for all of the 27 serial acquisitions in experiment 3 except those for the heart and epididymal fat due to significant blurring of these areas in the images and this data was not analyzed.

Statistical Analysis

All values are expressed as mean $\pm$ standard error of the mean (sem). A probability p value < 0.05 was considered significant to reject the null hypothesis. Body weights between scanning groups were tested using the Student's T -test. Linear regression analysis was performed (Prism 4) between R_1 relaxation values derived from MEMRI and tissue Mn concentration calculated by ICP-AES, and also dose-dependence of R_1 values in various organs. Percentage SI profiles in glucose challenge experiments were tested for significant differences using generalized estimating equations (GEE) using Stata 10 (Statacorp, Texas, USA).

Results

Experiment 1: Measurement of *in vitro* T_1 (and R_1) values by IR-SE and MP-RAGE

In this study, we have used the standard IR equation to fit SI data from MP-RAGE MRI to generate a surrogate measure of T_1 , T_1 -effective (T_1 -eff). The data is well described by the equation with a goodness of fit > 0.9958 (Supplementary Table 1) for the individual signal recovery curves at the various Mn^{2+} concentrations. T_1 -eff values were generally greater than the T_1 values at all Mn^{2+} concentrations (Figure 1A). Linear relationships were observed between both relaxivities measured by IR-SE and MP-RAGE and different $MnCl_2$ solutions (Figure 1B). In general, we observed an underestimation of relaxivities by MP-RAGE of $17.87 \pm 0.56 \%$, compared to by IR-SE.

Experiment 2: *In vivo* correlation between MP-RAGE and ICP-AES data following dose-dependent Mn^{2+} infusion in non-CNS organs.

Manganese administration led to general decreases in T_1 -eff values in the pancreas, kidney, liver, spleen and heart (Figure 2). Whilst in muscle and fat, T_1 -eff values remained unaffected even after infusion of high concentrations of $MnCl_2$ (Figure 2). This is consistent with the increased tissue Mn concentration as measured by ICP-AES (Figure 3). However, although a decrease in T_1 -eff was observed in the spleen, ICP-AES measurement of Mn was generally unchanged. The strong positive linear correlation between tissue Mn (as measured by ICP-AES) in the pancreas ($r^2 = 0.971$), kidney ($r^2 = 0.978$), liver ($r^2 = 0.923$) and heart ($r^2 = 0.960$) and infused $MnCl_2$, indicates dose-dependent Mn uptake (Figure 3). In addition, infusion of $MnCl_2$ results in greater Mn accumulation in the liver, kidney and pancreas compared to the heart (Figure 3). In the muscle, spleen and fat, infusion of up to 40mM $MnCl_2$ did not generally affect absolute concentrations of Mn as measured by ICP-AES ($p = NS$) (Figure 3). A linear correlation was observed between R_1 -eff values and Mn concentration in the pancreas ($r^2 = 0.7129$), kidney ($r^2 = 0.856$), liver ($R^2 = 0.753$) and heart ($r^2 = 0.807$) (Figure 4). No such correlate was observed for skeletal muscle and fat.

Experiment 3: The time-course effects of $MnCl_2$ infusion on SI in non-CNS organs

Prior to measurement of SI enhancement profiles in various organs, preliminary experiments were performed to determine the appropriate choice of the value of the flip angle for the MP-RAGE sequence. Data employing flip angles of 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50° is shown in supplementary Figure 1: significant image degradation was observed at the 50° flip angle and the data excluded. The differences in SI between pancreas and kidney, liver or spleen increased over the flip angle range 5-20° and then decreased for 20-40° (Supplementary Figure 1). SI of individual organs were compared with that of the pancreas as the latter is especially difficult to distinguish in the MR image. However, while differences in SI, as well as tissue SI were increased, image distortion was also increased with increasing flip angles, consistent with the work of Deichmann *et al.*, (Deichmann *et al.*, 2000). Thus, a flip angle of 10° was chosen as a compromise between high SI differences and low image blurring.

The tissue-specific, dose-response effects following i.v. infusion of MnCl₂ is shown in Figure 5. Liver, pancreas and kidney showed a marked increase in SI reaching a plateau around 30min, which was sustained for the duration of the scan. Increasing the MnCl₂ dose had a marked effect on the overall SI level, resulting in different saturation points for different tissues, e.g. 10mM in the liver (Figure 5C). In the skeletal muscle, low concentrations of MnCl₂ did not elicit any significant changes in SI. However, a transient increase in SI was observed at 40mM which returned back to baseline by 60min after the start of infusion (Figure 5D).

Discussions

It is well established that an increase in cytoplasmic Ca²⁺ induced by external stimuli leads to a wide range of effects in electrically excitable cells, such as neurons, muscle cells and pancreatic β-cells, as well as non-excitable cells such as lymphocytes, epithelial cells and endothelial cells (Knot *et al.*, 2005; Miura *et al.*, 1997). The absolute increase in cytoplasmic Ca²⁺ is a composite of both intracellular release from Ca²⁺ stores and Ca²⁺ entry from the extracellular space. Importantly, it is the entry of Ca²⁺ into the cell that drives most Ca²⁺-dependent responses (Parekh, 2006). In this respect, paramagnetic Mn²⁺ is an appropriate surrogate ion for assessment of Ca²⁺ influx during Ca²⁺ signaling processes by T₁-weighted MR imaging paradigms. Indeed, extracellular Mn²⁺ is taken up by a variety of different cell types which employ Ca²⁺-related processes, in a concentration- and time-dependent manner (Aoki *et al.*, 2006; Gimi *et al.*, 2006; Lee *et al.*, 2005; Rorsman *et al.*, 1982; Weed and Rothstein, 1960).

Mn²⁺ can be administered as either inorganic salts such as MnCl₂, or chelates such as manganese dipyridoxylidiphosphate (MnDPDP). MnCl₂ is often used as the MRI contrast agent in preclinical studies due to its rapid kinetics and low cost (Zheng *et al.*, 2000) whilst the chelated form of Mn²⁺ being less toxic, is more suitable for clinical practice. Plasma concentrations of Mn²⁺ return to baseline levels 1h after a single 100mM MnCl₂ i.v. injection (*unpublished observations*), compared to several hours following administration of the chelate (Krombach *et al.*, 2004; Sotogaku *et al.*, 2000). In our MEMRI studies, the use of controlled MnCl₂ infusion was chosen to avoid high peak serum levels of Mn²⁺ that can occur following bolus delivery (Silva *et al.*, 2004). Consequently, this limits the potentially adverse effects of this contrast agent on animal physiology and maximizes the duration of Mn²⁺ in the circulation for optimal cellular uptake. Moreover, previous studies have demonstrated MEMRI using a continuous MnCl₂ infusion for ~ 30 min can be successfully used to

detect neuronal activity (Kuo *et al.*, 2007; Parkinson *et al.*, 2009).

We have employed the standard T₁/R₁-measuring technique, IR-SE, demonstrating a linear correlation between R₁ and increasing MnCl₂ concentrations *in vitro*. Our data suggests that R₁ measurements can be used to accurately estimate Mn²⁺ concentration *in vitro*. A similar, positive correlation between R₁-eff values and Mn²⁺ concentration was also observed using MP-RAGE *in vitro*. Deviations in R₁-eff from the IR-SE linear model were observed by MP-RAGE assessment at very high Mn²⁺ concentrations in experiment 1 (see below). However, the R₁-eff values measured *in vivo* correlate strongly with absolute tissue Mn concentration in the pancreas, kidney, liver and heart as indicated by the ICP-AES data. This is of particular interest given that there is no histological staining protocol readily available for detection of Mn²⁺ in tissues *in vitro* (Bock *et al.*, 2008), let alone for *in vivo* assessment of tissue Mn²⁺ concentration. Our results suggest tissue Mn²⁺ concentration can be estimated *in vivo* according to its R₁-eff value. Therefore, whilst MP-RAGE may not provide an absolute measure of T₁, T₁-eff appears to serve adequately as a surrogate for both T₁ and tissue manganese concentration in experimental comparisons.

The equation describing the dependency of the MP-RAGE signal on T₁ is complex (24, 25). Jivan and colleagues simplified this equation to the IR equation based on certain criteria, including the employment of a low flip angle (< 8°) and an overall TR₀ of > 5T₁ (26). Here we have employed a flip angle of 10°, a compromise between minimizing image blurring and sufficient signal intensity and contrast. In order to compensate for this slightly higher flip angle, we employed centric rather than linear k-space filling; such that the MP-RAGE image contrast was weighted to the start of the acquisition window prior to possible perturbation of the longitudinal magnetization recovery by a subsequent RF pulse (20). In our *in vivo* studies the TR₀ (>3380ms) was selected as a compromise between having as long a TR₀ as possible while minimizing scan times. The TR₀ we selected is not greater than 5 times the T₁ in certain tissues as recommended by Jivan *et al.* (26). Further, we can address another source of the inaccuracy of MP-RAGE measurements by varying TR in order to account for the different T₁ values in measuring T₁. However, whilst we acknowledge these parameter values may be a source of error in our T₁ measurement by MP-RAGE, signal recovery in our studies is greater than 90 %; and indeed greater still when manganese is present.

The MP-RAGE data from experiment 3 shows significant increases in SI in most ROIs as the concentration of infused MnCl₂ increases, reflecting enhanced Mn²⁺ uptake within these tissues. An increase in SI occurred within the muscle at the highest MnCl₂ dose with no apparent effects on T₁-eff. This probably reflects the transient nature of the Mn²⁺ uptake in muscle relative to the T₁-measurement window. In tissues exhibiting positive enhancement, the percentage change in SI tended to reach a maximum 30min from the start of Mn²⁺ infusion which was then sustained for the duration of the scan. With regard to application of MP-RAGE MEMRI to the measurement of calcium channel activation, it should be noted that at the time of T₁ measurement, post a 30min infusion, there is likely to be Mn²⁺ present in the plasma, interstitial tissue and intracellular space. This is an issue for the majority of all MEMRI techniques, where intracellular constituents are responsible for changes in SI. This possible confounder to accurate quantification of Ca²⁺ channel activity using may be addressed by additional non-MRI studies examining the time-dependent nature of Mn²⁺ uptake.

It should be noted that the highest dose of MnCl_2 we have used does not appear to have reached a threshold saturation point for Mn^{2+} uptake in the pancreas, kidney, liver or heart (Figure 3A) suggesting these organs have a greater capacity for Mn^{2+} uptake. In agreement with previous work (Kuo et al., 2005), we have observed dose-dependent SI increases in the pancreas, kidney and liver. Given the importance of balancing MRI signal optimization and minimizing toxicity (Kuo et al., 2007; Silva et al., 2004), this data provides a practical MnCl_2 dosing regimen for use in tissue-specific activation studies. Furthermore, tissues with similar Mn^{2+} accumulation patterns may reflect similar physiological properties, such as arterial input function, and could also act as a preferable internal reference in subsequent MEMRI scans.

There are however, inherent limitations to the MEMRI technique that should be addressed. Firstly, current MRI methodologies are not capable of operating at sub-cellular resolutions and therefore, the process of redistribution of intracellular Ca^{2+} stores cannot be detected by MEMRI. Mn^{2+} is also toxic and tissue Mn^{2+} levels need to generate adequate SI for MRI but not significantly affect cell viability or function (Silva et al., 2004). Whilst MEMRI analysis is capable of generating a quantitative measure of a response to a stimulus, it is unable to discriminate between differences in activity of different cellular populations within the same ROI.

In conclusion, we have shown that MP-RAGE MRI is an effective means of determining the distribution of Mn^{2+} in non-brain organs following i.v. infusion, and the estimated T_1 values aid determination of optimal MRI parameters to be employed at 4.7T. The dose-dependent Mn^{2+} distribution in the major non-brain organs in mice provides a practical MnCl_2 dosing regimen for use in tissue-specific activation studies. More work will be required to further the application of MEMRI to measure activation in electrically non-excitable cells in which Mn^{2+} has been proven to act as an indicator for Ca^{2+} fluxes, such as platelets (Hallam and Rink, 1985; Rink and Sage, 1990), lymphocytes (Balasubramanyam et al., 1993), endothelial cells (Hallam et al., 1988), neutrophils (Merritt et al., 1989), parotid acinar cells (Mertz et al., 1990), adrenal chromaffin cells (Powis et al., 1996) and mast cells (Fasolato et al., 1993). This study provides a potential 3D MRI technique for *in vivo* imaging of Ca^{2+} entry during Ca^{2+} -dependent processes in a wide range of tissues, not just in the brain.

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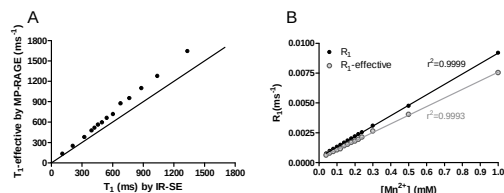


Figure 1. (A) Comparison of T_1 and T_1 -eff values obtained by IR-SE and MP-RAGE, respectively, (a line of equivalence is also displayed). (B) Linear correlation analysis between Mn^{2+} concentrations and R_1 or R_1 -eff relaxivities. Data analysed using Prism 4, (GraphPad, USA).

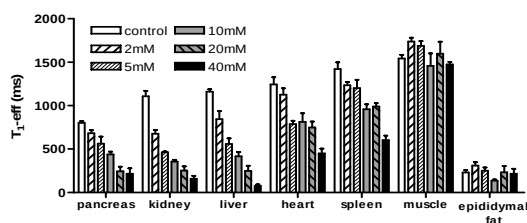


Figure 2. Changes in T_1 -eff values of various non-CNS tissues following i.v. infusion of 0.1ml of $MnCl_2$ (2, 5, 10, 20 or 40mM) ($n = 3$ /group). T_1 -eff values were recorded following a 30min $MnCl_2$ infusion. The control group

received no infusion ($n = 6$). Data are presented as mean $\pm$ sem.

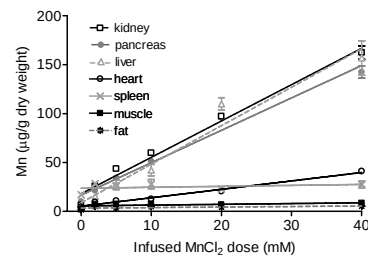


Figure 3. Tissue-specific relationship between infused $MnCl_2$ (0.1ml of 2, 5, 10, 20 or 40mM dose) and manganese content as determined by ICP-AES in various mouse organs. Animals were euthanized following a 30min i.v. infusion of $MnCl_2$ and 22.5min of MP-RAGE based MRI scanning. First order linear regression analysis was performed using Prism 4 (GraphPad, USA). Data are presented as mean $\pm$ sem.

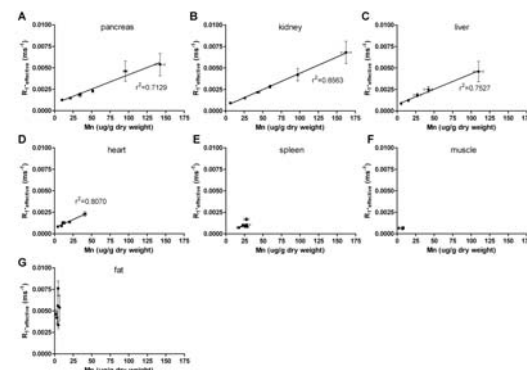


Figure 4 Tissue-specific relationship between R_1 -eff values and manganese content as determined by ICP-AES in various non-CNS organs; (A) pancreas, (B) kidney, (C) liver, (D) heart, (E) spleen, (F) muscle and (G) fat. First order linear regression analysis was performed using Prism 4 (GraphPad, USA). Data are presented as mean $\pm$ sem.

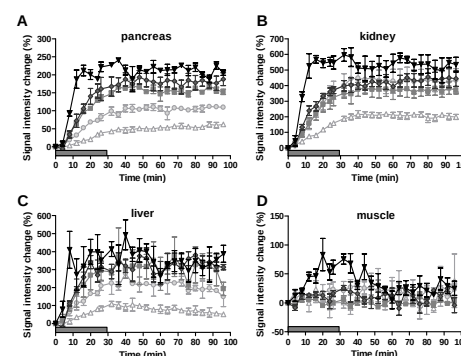


Figure 5. The percentage change in SI from baseline in (A) pancreas, (B) kidney, (C) liver and (D) muscle, following i.v. infusion of various doses of $MnCl_2$ (2, 5, 10, 20 or 40mM, 0.2 ml/h); the grey box indicates the duration of manganese infusion. $n = 3$ /group. Data are presented as mean $\pm$ sem.