Oxytocin and its effect on the pathological role of the AGE-RAGE complex in cardiovascular complications of type I and II diabetes mellitus

By

Denis Yip

Department of Experimental Medicine

Faculty of Medicine

McGill University

Montreal, Quebec, Canada

July 2014

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Abstract

Introduction: There is significant mortality in the diabetic population due to cardiovascular complications. One of the characteristic biochemical changes often found in diabetes is the formation of advanced glycation end products (AGEs). AGEs are one of the primary ligands for the receptor of AGE (RAGE) and have previously been implicated in the initiation and maintenance of a pathological pro-inflammatory response primarily through NF-κB. The AGE-RAGE interaction has been directly linked to deleterious effects eventually leading to pathologies such as atherosclerosis and cardiomyopathy. Oxytocin, a peptide hormone, which induces parturition, has been shown to have cardioprotective properties. Activation of the oxytocin receptor can induce the shedding of RAGE thereby decreasing the inflammatory response.

Objective: To identify differences in the metabolic and cardiac function by oxytocin treatment in animal models of type I and II diabetes mellitus and to explore the protective mechanism achieved following oxytocin therapy in both models.

Methods: Experiments were performed in type I and II diabetic animal models and in H9c2 embryonic rat cardiac myoblasts. Four-week old Wistar rats were treated with a single dose of streptozotocin (65 mg/kg) to induce type I diabetes after two days, followed by oxytocin infusion (125 ng/kg/h) using osmotic Alzet pumps for six weeks. Similarly, four-week old *db/db* mice of type II diabetic phenotype were treated with oxytocin (125 ng/kg/h) for 12 weeks. After sacrifice, the heart and aorta were collected from control (non-diabetic), diabetic and oxytocin treated animals then stained for accumulation of collagen and polysaccharides. Protein levels were measured by Western blot. H9c2 cells were pre-treated with glyceraldehyde-AGE (100-400 μg/mL) for two hours followed by oxytocin (62.5 nM and 300 nM) for five hours. Cell viability was measured by a resazurin assay and protein expression through in-cell ELISA.

Results: Oxytocin administration diminishes body weight changes and glycemia in type I and II diabetic animals. Heart weight was unaffected by oxytocin treatment in type I diabetic rats nor in type II diabetic mice. RAGE expression was also found to be significantly reduced upon oxytocin treatment in both type I diabetic (3.56 ± 1.01 fold vs. 1.10 ± 0.40 fold in treated

animals, $p<0.05$) and type II diabetic (1.55 ± 0.05 fold vs. 1.13 ± 0.01 fold in treated animals, $p<0.001$). The expression of NF-κB was not elevated in type I diabetic rats but increased in type II diabetic mice. Elevation of NF-κB was associated to the increases in reactive oxygen species accumulation and reduction of cell apoptosis seen in the untreated *db/db* mice. These effects were reversed by oxytocin treatment. The treatment of H9c2 cells with AGEs did not present any effect on cell viability in a dose or time dependent manner. Cells pre-stimulated with AGEs showed significantly increased levels of phosphorylated NF-κB ($p<0.05$) but no changes in total NF-κB. Treatment with oxytocin also did not appear to decrease total levels of NF-κB or the phosphorylated NF-κB levels.

Conclusions: Oxytocin administration in diabetic animals can ameliorate cardiac metabolic changes associated with diabetes. Protection against activation of the inflammatory pathway including the increased production of reactive oxygen species and cell apoptosis is more effective in models of type II diabetes compared to the model of type I diabetes.

Abrégé

Introduction: Une mortalité significative est observée chez la population diabétique en raison de complications cardiovasculaires. Un des changements biochimiques caractéristiques souvent observés chez les personnes atteintes de diabète est la formation de produits de fin de glycation avancée des protéines (AGEs). Les AGEs, un des ligands primaires pour le récepteur de l'AGE (RAGE), avaient été associés auparavant à l'initiation et le maintien des réponses pathologiques pro-inflammatoires principalement par NF-κB. L'interaction AGE-RAGE avait été directement liée aux effets délétères, menant éventuellement à des pathologies, telles que l'athérosclérose et la cardiomyopathie. Il a été démontré que l'ocytocine, une hormone peptidique qui induit la parturition, a des propriétés cardio-protectrices. L'activation du récepteur de l'ocytocine pourrait induire un 'clivage' du RAGE, diminuant ainsi la réponse inflammatoire.

Objectif: Identifier les différences dans les fonctions cardiaques et métaboliques par le traitement de l'ocytocine chez des modèles animaux de diabètes de types 1 et 2 et explorer les mécanismes protecteurs obtenus à la suite d'une thérapie à l'ocytocine dans les deux modèles.

Méthodes: Les expériences ont été réalisées sur des modèles animaux de diabète de types 1 et 2 et des myoblastes cardiaques d'embryons de rat H9c2. Des rats Wistar, âgés de quatre semaines, ont été traités avec une dose de streptozotocine (65 mg/kg) pour induire le diabète de type 1 après deux jours, puis une perfusion continue d'ocytocine (125 ng/kg/h) a été administrée pendant six semaines dans des pompes osmotiques Alzet. Similairement, des souris diabétiques de type 2 *db/db* âgés de quatre semaines, ont été traitées avec l'ocytocine (125 ng/kg/h) pendant 12 semaines. Après le sacrifice, le coeur et l'aorte sont recueillis d'animaux contrôles (non diabétiques), diabétiques et d'animaux traités avec l'ocytocine, puis sont marqués pour l'accumulation de collagène et de polysaccharides. L'expression des protéines est évaluée par Western blot. Les cellules H9c2 étaient prétraitées avec du glycéraldéhyde-AGE (100-400 μg/mL) pendant deux heures, puis avec l'ocytocine (62,5 nM et 300 nM) pendant cinq heures. La viabilité cellulaire a été mesurée par un test de résazurine et l'expression des protéines phosphorylées par un test ELISA.

Résultats: L'administration d'ocytocine diminue le poids corporel et la glycémie dans les modèles animaux de diabète (types 1 et 2). Le poids du coeur n'a pas été affecté par le traitement avec l'ocytocine chez les rats diabétiques (type 1) ni chez les souris diabétiques (type 2). L'ocytocine a réduit significativement l'expression de RAGE chez les animaux diabétiques aussi bien avec diabète de type 1 ($p<0,05$) que de type 2 ($p<0,001$). L'expression de NF-κB était élevée chez les souris diabétiques de type 2 mais pas chez les rats diabétiques de type 1. L'élévation de NF-κB était associée à l'augmentation de l'accumulation de dérivés réactifs de l'oxygène et à la diminution de l'apoptose cellulaire observée chez les souris *db/db* non traitées. Ces effets ont été inversés par un traitement avec l'ocytocine. Le traitement des cellules H9c2 avec AGEs n'a pas affecté la viabilité cellulaire aussi bien avec les doses que les durées de stimulation testées. Les cellules préstimulées avec AGEs montraient des niveaux significativement augmentés de NF-κB phosphorylés ($p<0,05$) mais NF-κB total n'est pas affecté. Le traitement avec l'ocytocine ne semble pas diminuer les niveaux totaux de NF-κB ou les niveaux de NF-κB phosphorylé.

Conclusions: L'administration d'ocytocine aux animaux diabétiques peut améliorer les changements cardio-métaboliques associés au diabète. La protection contre l'activation de la voie inflammatoire incluant une augmentation de la production des dérivés réactifs de l'oxygène et l'apoptose cellulaire est plus efficace dans le modèle du diabète de type 2 comparé au diabète de type 1.

Acknowledgements

I would like to thank my supervisor, Dr. Jolanta Gutkowska, for accepting me into her laboratory and guiding me through my Master's project. Your devotion to science has instilled upon me the importance of persistence and patience.

I would also like to express my deepest gratitude to my thesis committee member, Dr. Marek Jankowski, whose expertise and extensive knowledge has inspired my work and given me the encouragement to persevere through my studies. Your continued support has contributed to my success in the field of science and shown me my potential.

I would also like to thank the members of my lab, Dr. Ahmed Menaouar, Dr. Eric Plante and Dr. Bogdan Danalache. Your motivation and technical expertise has helped me progress through my project.

Araceli Gonzalez-Reyes and Sophie Annabelle Barrateau, thank you for the moral and emotional support that you have provided me throughout the past few years. Your advice has been invaluable and has helped me through some of my toughest times. And to my friends, Smitha, Christine and Darshana, who have put up with me through the long nights and pushed me through the final stretch.

Lastly, I would like to thank my parents, brother and grandmother, who have stood by me through the best and the worst of times. You have all been an inspiration to me.

Dedication

This thesis is dedicated to my parents whose love, support and encouragement has helped me through the challenges in my life and to my grandmother who has always driven me to persevere.

Table of Contents

Dedication vii

Table of Contents viii

List of Tables xi

List of Figures xii

List of Abbreviations xiii

1. Introduction 1

1.1 Diabetes Mellitus 1

1.1.1 Classification of Diabetes 2

1.1.2 Symptoms of Diabetes 2

1.1.3 Current Therapeutic Intervention for Diabetes Mellitus 3

1.1.4 Animal Models of Diabetes 4

1.2 Cardiovascular Disease in Diabetes 6

1.3 Advanced Glycation End-Products 7

1.3.1 Mechanism of AGE Generation - Maillard Reaction 8

1.3.2 Development in Diabetes 10

1.4 Receptor of Advanced Glycation End-products 10

1.4.1 RAGE Isoforms 11

1.4.2 Receptor Activation 12

1.4.3 Signalling Cascade & Downstream Pathways 13

1.5 Inflammation in Diabetes 14

1.5.1 Implications in Diabetes 14

1.5.2 AGEs & NF-κB 15

1.6 Oxidative Stress 16

1.6.1 Role in Inflammation 17

1.6.2 Oxidative Stress in Diabetes 17

1.7 Oxytocin 18

1.7.1 Oxytocin Receptor & Action of Oxytocin 20

1.7.2 Effects of Oxytocin on CVD 21

1.7.3 Effects of Oxytocin on DM 22

1.8 Hypothesis 24

1.9 Research Aims 24

2. Materials & Methods 25
2.1 Animal Models 25
2.1.1 Streptozotocin-induced Diabetic Rats (Type I Diabetes) 25
2.1.2 *db*/*db* Diabetic Mice (Type II Diabetes) 25
2.1.4 Periodic Acid Schiff Staining of Aorta 26
2.1.5 Western Blot of Left Ventricle 26
2.1.6 Reactive Oxygen Species Accumulation 29
2.1.6 TUNEL Assay of Cellular Apoptosis 30
2.1.7 Study Approval 30
2.2 Cell Culture 31
2.2.1 H9c2 Cell Line 31
2.2.2 *In vitro* glyceraldehyde-derived AGE Generation 31
2.2.3 PrestoBlue Evaluation of Cell Metabolism 32
2.2.4 In Cell ELISA 33
2.3 Statistical Analysis 34
3. Results 35
3.1 *In vivo* Results 35
3.1.1 Metabolic effects of oxytocin 35
3.1.2 Evaluation of Cardiac Weight 40
3.1.3 DM modifies protein expression of RAGE and downstream pro-inflammatory factors 42
3.1.4 DM may amplify the accumulation of reactive oxygen species in left ventricular tissue 46
3.1.5 Apoptosis of cardiac cells in diabetic animal models 48
3.2 *In vitro* Results 50
3.2.1 Evaluation of *in vitro* generation of glyceraldehyde-AGE 50
3.2.2 Assessment of AGE product stability in a time-dependent manner 51
3.2.3 Viability of H9c2 cells following AGE treatments 53
3.2.4 Effects of AGE stimulation on activation of NF-κB 56
3.2.5 Oxytocin post-treatment on NF-κB protein levels following AGE stimulation 59
4. Discussion 61
4.1 Oxytocin reverses metabolic and morphological changes caused by diabetes mellitus 61
4.1.1 Oxytocin protects against metabolic changes 61
4.1.2 Cardiac implications and vascular morphology reversal by oxytocin treatment 62
4.2 RAGE is a key player in the inflammatory process found in type I and II diabetes mellitus 64

4.3. Exogenous oxytocin administration reverses the upregulation of RAGE expression 66
4.4 Disruption of RAGE signalling cascade reduces reactive oxygen species accumulation and apoptosis in models of type II diabetes but not in type I diabetes 68
4.5 Protective mechanisms activated by oxytocin 69
4.6 Current therapeutics for inflammation 71
5. Conclusions 72
6. Supplementary Material 73
7. References 74

List of Tables

Table 1: Categories of Advanced Glycation End-products 7

Table 2: Major isoforms of the receptor of advanced glycation end-products 12

Table 3: Inflammatory cytokines implicated in development of diabetes mellitus 14

List of Figures

Figure 1: Structure of oxytocin and arginine vasopressin. 20

Figure 2: Body weight of control and diabetic animals. 36

Figure 3: Glycemic management of blood glucose levels. 37

Figure 4: Effect of oxytocin treatment on oral glucose tolerance test in diabetic models. 39

Figure 5: Heart weight of STZ and *db*/*db* animals. 41

Figure 6: RAGE expression in the left ventricle of diabetic animals. 43

Figure 7: Protein expression of phosphorylated NF-κB and total NF-κB in the left ventricle of diabetic animals. 45

Figure 8: Immunohistochemical staining of reactive oxygen species accumulation in left ventricular tissue of type I and type II diabetics animals. 47

Figure 9: Cell apoptosis in left ventricle marked in models of type I and II diabetes. 49

Figure 10: Fluorescence curve of *in vitro* generated glyceraldehyde-advanced glycation end products. 51

Figure 11: Assessment of *in vitro* glyceraldehyde-advanced glycation end-products stability. 52

Figure 12: Dose dependent cell viability assessment of H9c2 cells treated with AGEs. 54

Figure 13: Cell viability test of H9c2 cells treated with AGEs in a time dependent manner. 55

Figure 14: NF-κB protein expression through time dependent AGE stimulation of H9c2 cells. 57

Figure 15: NF-κB protein expression through dose dependent AGE stimulation of H9c2 cells. 58

Figure 16: NF-κB protein expression following a dose dependent AGE stimulation and oxytocin treatment. 60

Figure 17: Mechanism of anti-inflammatory action by oxytocin. 67

List of Abbreviations

ADAM10- a disintegrin and metalloproteinase domain containing protein 10

AGE- advanced glycation end-product

Akt- protein kinase B

AMPK- 5' adenosine monophosphate activated protein kinase

ANOVA- analysis of variance

AVP- arginine vasopressin

BB- bio-breeding

BCA- bicinchoninic acid

BSA- bovine serum albumin

Ca^{2+}- calcium

CaMKII- Ca^{2+}/calmodulin-dependent protein kinase II

CCAC- Canadian Council of Animal Care

Cdc- cell division control protein

CRP- C-reactive protein

CVD- cardiovascular disease

DAB- 3,3'-diaminobenzidine

DAG- 1,2 diacylglycerol

DAG-PKC- diacylglycerol-protein kinase C

DAPI- 4',6-diamidino-2-phenylindole

DKA- diabetic ketoacidosis

DM- diabetes mellitus

DMEM- Dulbecco's Modified Eagle's Medium

DNA- deoxyribonucleic acid

DNPH- 2,4-dinitrophenylhydrazine

dnRAGE- dominant negative receptor of advanced glycation end-products

eNOS- endothelial nitric oxide synthase

ERK- extracellular signal regulated kinase

esRAGE- endogenous secretory receptor of advanced glycation end-products

FBS- fetal bovine serum

FH1- formin homology domain-1

GAD- glutamic acid decarboxylase

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

GPCR- G-protein coupled receptor

GTP- guanosine triphosphate

HbA1c- glycated hemoglobin

HDL- high density lipoprotein

HEK- human embryonic kidney

HMGB1- high mobility group box-1

HRP- horseradish peroxidase

ICAM-1- intercellular adhesion molecule-1

IDDM- insulin dependent diabetes mellitus

IκB- inhibitor of κB

IL- interleukin

iNOS- inducible nitric oxide synthase

IP- inositol triphosphate

Jak-STAT- janus kinase and signal transducer and activator of transcription

kDa- kilodaltons

LDL- low density lipoprotein

MAP- mitogen activated protein

mDia1- diaphanous-1

MHC- major histocompatibility complex

MMP- matrix metalloproteinase

MnSOD- manganese superoxide dismutase

MyD88- myeloid differentiation primary response gene 88

NF-κB- nuclear factor kappa-light chain-enhancer of activated B cells

NIDDM- non-insulin dependent diabetes mellitus

nNOS- neuronal nitric oxide synthase

NO- nitric oxide

NOD- non-obese diabetic

NOS- nitric oxide synthase

NSAID- non-steroidal anti-inflammatory drug

NtRAGE- N-truncated receptor of advanced glycation end-products

OGTT- oral glucose tolerance test

OTR- oxytocin receptor

PBS- phosphate buffered saline

PI3K- phosphatidylinositol-3-kinase

PKC- protein kinase C

Rac- Ras-related C3 botulinum toxin substrate

RAGE- receptor of advanced glycation end-products

RFU- relative fluorescent units

RNA- ribonucleic acid

RNS- reactive nitrogen species

ROS- reactive oxygen species

SDS- sodium dodecyl sulphate

SERCA2a- sarcoplasmic/endoplasmic reticular Ca^{2+}-ATPase 2a

SHP-1- Src homology-2 domain-containing phosphatase-1

sRAGE- soluble receptor of advanced glycation end-products

STZ- streptozotocin

TBS- Tris-buffered saline

TBS-T- Tris-buffered saline with Tween

TIRAP- toll-interleukin 1 receptor domain containing adaptor protein

TNF- tumour necrosis factor

Tris- tris(hydroxymethyl)aminomethane

TUNEL- terminal deoxynucleotidyl transferase dUTP nick end-labeling

UCP-1- uncoupling protein-1

UTR- untranslated region

VCAM-1- vascular cell adhesion molecule-1

VEGF- vascular endothelial growth factor

WHO- World Health Organization

1. Introduction

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a complex metabolic disorder that is characterized by various symptoms but is best known through the development of hyperglycaemia resulting from a defect in the insulin mechanism [1]. Historically, it was not considered to be a disease of great concern but in the present day, it is becoming better diagnosed. The increasing prevalence of DM is placing a major strain onto worldwide health systems and becoming a global epidemic [2]. Changes to social and environmental surroundings have caused a surge in factors that influence the development of DM. Global industrialization has progressively led to more sedentary lifestyles prompting a rise in rates of obesity worldwide [3, 4]. Our inability to keep up and adapt to the fast-paced changes to our work and life habits advocate for a more idle and inactive living style that encourages the development of DM. It is becoming clear that the increase in individuals suffering from pre-diabetes and DM show no sign of declining but will only continue to escalate as the baby boomers age [5]. As of 2014, it is estimated that about 25 million people in the United States have type II diabetes, and another million have type I diabetes [6]. In a recent study, it has been predicted that in the United States, rates of DM will increase from the current trend of 1 in 10 individuals suffering from DM to an astounding rate of 1 in 5 to 1 in 3 by the year 2050 [5]. In 2008/09, almost 2.4 million Canadians (6.8%) were living with diabetes [7]. For this reason, it is imperative to gain a strong understanding of both the aetiology and symptoms so that new therapeutic options can be explored.

1.1.1 Classification of Diabetes

DM has previously fallen into two main categories through observations of insulin sensitivity performed by Himsworth in 1936 [8]. DM type I and II were formerly referred to as insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) respectively, based solely on the clinical presentation regardless of aetiology. In 1998, this classification of DM was abolished over preference for a new set of classifications with DM type I being reclassified by the World Health Organization (WHO) as "insulin requiring for survival" [9]. Type I DM most commonly refers to individuals suffering from DM due to pancreatic β-islet cell death who have a greater tendency to develop ketoacidosis due to a lack of insulin secretion [9]. As a result, patients who suffer from DM type I are often prescribed exogenous insulin as a preventative measure against the progression to diabetic ketoacidosis (DKA) [2].The development of type I DM is primarily of autoimmune or of idiopathic nature [9]. Comparatively, DM type II was subdivided into two categories, "insulin requiring for control" and "not insulin requiring". Today, type II DM is the most common form of diabetes making up greater than 90% of cases [2, 9]. Type II DM is characterized by insulin resistance and/or abnormal insulin secretion [2]. For this reason, patients diagnosed with type II DM are not dependent on insulin but may rely on its use for management of their blood glucose levels.

1.1.2 Symptoms of Diabetes

DM can present a number of symptoms ranging from mild (i.e. thirst, polyuria, and blurry vision) to more severe such as coma and death if left untreated [9]. At times, patients with DM may be asymptomatic or present with mild symptoms that are not severe. This may lead to increased cases of undetected DM until the chronic hyperglycemic state has caused functional

changes and/or damage to the organs. During this asymptomatic period, a diagnosis of DM can be made through different clinical tests such as a measurement of oral glucose tolerance or by measuring the fasting plasma glucose levels. The long-term pathogenic effects of DM are often exacerbated in particular sites of the human body such as the kidneys, nerves, eyes, blood vessels and the heart with kidney nephropathy, peripheral neuropathy, retinopathy and cardiovascular problems being key concerns respectively [10].

1.1.3 Current Therapeutic Intervention for Diabetes Mellitus

There is a growing need for innovative therapeutic techniques to combat both the development of DM and its associated complications. Currently, treatment of type I DM requires the administration of exogenous insulin to regulate a patient's blood glucose levels preventing the development of DKA. The administration of insulin has markedly improved over the years and can now be performed via a traditional syringe, insulin pen or an insulin pump. Often times, the patient's therapy can be customized to their lifestyle to better suit their needs [11]. In recent years, there has been interest in immune system mediation as a therapeutic technique to delay the onset of auto-immune islet cell destruction found in type I DM. Studies have identified the role of auto-antigens on glutamic acid decarboxylase (GAD), insulin and proinsulin, protein phosphatases as well as peptides of heat shock proteins in the development of type I DM [12–14]. Some clinical trials have reported promising results in the delivery of auto-antigens as an inducer of immune self-tolerance while others have proven to be ineffective [14]. One clinical trial studying immunizations against GAD auto-antibodies presented discouraging results with those receiving either placebo or GAD immunization continuing to develop reduced insulin production indicative of failing β-islet cells [15]. Further study of auto-immune regulation is

critical in the generation of novel therapeutics against type I DM. It is important to understand the complexity of the immune system and the need for target specificity without compromising a patient's overall immune response.

Compared to type I DM where insulin is considered to be first line therapy; the suggested initial approach for treatment of type II DM is lifestyle modifications prior to pharmacotherapy. Upon failure of lifestyle changes in maintaining glycemic levels, anti-hyperglycemic therapy is started. First line pharmacological therapy is controversial and is dependent on the patient's initial blood glucose level at the time of diagnosis. General recommendations suggest administration of oral metformin due to its ability to drastically lower blood glucose, its benefits on the cardiovascular system and its minimal side effects [16]. Alternatives include monotherapy with thiazolidinediones that provide better glucose control but tend to have greater side effects. Combination therapy of anti-hyperglycemics and insulin administration may be preferred and can be personalized to individual patients for optimal glucose control [17].

1.1.4 Animal Models of Diabetes

The upward trend of DM worldwide stimulates research on the aetiology and pathology of the disease. Experiments on cell culture and animal models provide tools that can be used to further the understanding of molecular pathways and the development of therapeutic agents in humans [18–20].

Some models used for the study of type I DM are generated through chemical induction or spontaneously through genetic modifications. One of the methods to induce type I DM is through the use of streptozotocin (STZ), a nitrosourea derivative from *Streptomyces achromogenes*, which interferes with glucose transporters and causes DNA breaks.

Streptozotocin can be administered either as a large, single dose or alternatively as multiple small doses to cause an immune response leading to destruction of pancreatic ß cells [21]. There are also two genetically modified models commonly used for studying type I DM because of their similarities to human pathology, the non-obese diabetic (NOD) mouse model and the bio-breeding (BB) rat model [21, 22]. The in-bred NOD model is useful in studying type I DM without the confounding effects of a heterogenous population and the BB rat develops symptoms on a timeline more comparable to humans [21].

The use of animal models is not limited to the study of type I DM but also useful in type II DM. Type II DM is a disease involving many environmental and genetic factors making it difficult to imitate the pathophysiological symptoms seen in humans. The most important criteria that is required for a model of type II DM is the ability for the animal to develop obesity and eventually diabetes [22]. Some of the better-known models that are currently used include leptin deficient (*ob*/*ob*) or leptin receptor activity deficient mice (*db*/*db*) and rats (Zucker) [22]. The *db*/*db* mice display hyperphagic and hyperinsulinemic tendencies with greater hyperglycemia than *ob*/*ob* mice [23]. The Zucker rats are comparable to *db*/*db* mice with the development of accompanying hyperlipidemia and hypertension. Other models exist that better resemble the conditions of type II DM but are not optimal for cardiac studies due to their delays in developing cardiac pathology. A recent study by Omar et al. [24], suggests that the use of middle aged mice on a high fat diet over one year better demonstrates the pathologies described in humans. Some animal models fed high fat, high sucrose diets may not develop cardiac dysfunction until up to two years following the start of the diet [25].

1.2 Cardiovascular Disease in Diabetes

One of the growing concerns in today's society is the development of cardiovascular disease (CVD). In 1998, a report found that approximately 30.9% of deaths worldwide were linked to CVD, followed by cancer related deaths at 13.4% of deaths [26]. High risk factors for the development of CVD include hypertension, obesity and diabetes. The role of diabetes in CVD has previously been outlined in the Framingham Heart Study noting that CVD was occurred at a two to three times greater incidence in diabetics than non-diabetics [27]. Mortality associated with diabetes was largely caused by coronary heart disease. DM can trigger the development of CVD through pathways such as protein modifications, dyslipidemia and changes in levels of plasma fatty acids [28]. The development of atherosclerotic plaque and in later stages, the process of plaque rupture can lead to the clinical symptoms that are observed in CVD.

Protein modifications through a process known as glycation can be used as a marker of DM. Modifications of haemoglobin through this process (HbA1c) is commonly used to monitor an individual's glycemic control [28]. Other protein modifications may lead to generation of advanced glycation end-products (AGEs) and oxidized low-density lipoproteins (LDL). These proteins have been implicated in accelerating the development of atherosclerosis [29, 30]. Dyslipidemia is characterized by an abnormal lipid profile with elevated triglycerides and LDL as well as lowered high-density lipoproteins (HDL). LDL proteins are more vulnerable to oxidation and are considered to be pro-atherogenic [31]. Further adding to insult is the propensity for high plasma fatty acid concentrations in diabetics. This condition can impair insulin signalling and disrupt glucose uptake into muscle. Rises in plasma fatty acids will also affect basal levels of nitric oxide (NO), a key component for maintaining normal endothelial function [32].

It is becoming increasingly more crucial to understand the mechanisms behind the development of CVD. Advancing current drug interventions for prevention and treatment relies heavily on understanding disease development and progression. The use of animal and cell culture models to mimic diabetic and cardiovascular conditions is invaluable.

1.3 Advanced Glycation End-Products

Advanced glycation end-products are a set of complex and heterogenous molecules that exist endogenously in the human body and exogenously in the environment such as in diet [33, 34]. These products are not well characterized leaving much of their mechanistic behaviour to be unclear. The existence of AGEs have displayed a large diversity of characteristics which include but are not limited to the ability to cause protein cross-linking, browning and auto-fluorescence. These characteristics, protein cross-linking in particular, may play a significant role in the development of pathology. In normal physiological conditions, the generation of AGEs occurs through the ordinary aging process. But, there are certain conditions that can contribute to and expedite the formation of AGEs including diabetes and smoking. Through the years, different variations of AGEs have been identified and are classified into 3 categories (Table 1) [34].

Table 1: Categories of Advanced Glycation End-products

Classification	Examples
Fluorescent, cross-linking AGEs	Pentosidine Crossline
Non-fluorescent, cross-linking AGEs	Imidazolium dilysine cross-links Alkyl formyl glycosyl pyrrole cross-links Arginine lysine imidazole cross-links
Non-cross-linking AGEs	Pyrraline N-carboxymethyllysine

Endogenous AGEs exist in the circulation of the human body and have been known to play a role on macromolecular structures and functions. Previously, it was thought that the existence of endogenous AGEs primarily affected proteins with long half lives, but it is now recognized that they also have an affect on structures of short life spans as well [33, 34]. With this observation, it has been found that AGEs can cause modifications on plasma and cytoplasmic proteins, lipids and nucleic acids [35]. The changes made to the proteins can alter its intended activity, modify its degradation as well as reduce recognition and clearance. The pathological effect of AGEs on short-lived molecules is well documented in lipids and lipoproteins. Similarly, AGE modifications of molecules with longer life spans may contribute to other pathologies [35]. It has also been found that AGEs in the circulation are able to activate the immune response allowing for the production of auto-antibodies against AGEs found in the blood serum. Generation of these antibodies may lead to further immune activation and clustering of the AGE-antibody conjugates which promote atherogenesis [34].

The exogenous existence of AGEs are most commonly found in our diets and through smoking. Due to the complex nature of AGE production and its signalling mechanism, it is not well understood how the administration of exogenous AGEs contributes to its pathological effects [33].

1.3.1 Mechanism of AGE Generation - Maillard Reaction

During the metabolism of nutrients such as carbohydrates, lipids and proteins there is a constant production of reactive carbonyl compounds [36]. The breakdown of these essential nutrients causes a number of biochemical processes to occur producing AGEs. The process is

complex and requires the completion of a series of reactions referred to as the Maillard reaction. The Maillard reaction is a set of reactions that are common to the production of many food products and involve a non-enzymatic browning process such as that found in the browning when baking bread. The glycation process is slow and is usually completed over a period of weeks to months [33].

The overall reaction can be broken down into 3 general stages. The process begins through the initial nucleophilic reaction of an aldehyde or ketone of a reducing sugar (i.e. glucose) covalently binding to a free amino group [36]. The resulting product is referred to as a Schiff base. The Schiff base is unstable and will spontaneously undergo a molecular rearrangement through cyclization and isomerization to generate a ketoamine known as Amadori's product [36, 37]. Up to this point, although the aforementioned reactions are reversible, the Schiff base rearrangement to Amadori's product strongly favours the product making the process difficult to reverse once it has occurred. From this, the Amadori's products are then able to degrade through autocatalysis or other chemical reactions into highly reactive carbonyl compounds, α-dicarbonyls and oxoaldehydes, which include 3-deoxy-glucosone, glyoxal or methyl-glyoxal leading to the generation of intermediate glycation products. These intermediate products that are generated and accumulated can lead to “carbonyl stress” [33]. The intermediate compounds undergo further chemical reactions and polymerization to generate the heterogenous AGE products. Alternatively, the carbonyl compounds may independently cause biological effects or go through further, slower changes via a different pathway that will ultimately lead to the final generation of AGEs [36, 37].

Since the initial stages of AGE formation are reversible, there are attempts to develop new therapeutics that are able to interrupt the formation of AGEs as well as some efforts in preventing protein cross-linking.

1.3.2 Development in Diabetes

In DM, high blood sugar levels are a significant contributor to the development of AGEs as it provides a very favourable environment for their generation. AGE formation is dependent on many factors including the degree of hyperglycaemia, the half-life of the protein and the permeability of the tissue to glucose [34]. The generation and accumulation of AGEs in a diabetic setting has been found to be harmful to the overall well being of the individual. Clinical studies of AGE inhibitors (i.e. aminoguanidine) have the ability to reduce diabetic complications which corroborates the role of AGEs in DM [38].

1.4 Receptor of Advanced Glycation End-products

The receptor of advanced glycation end-products (RAGE) is a highly conserved multi-ligand receptor that belongs to the immunoglobulin superfamily of cell surface molecules [39–41]. The receptor was first discovered in 1992 by Schmidt et al. [42] in bovine endothelial cells and lung extracts when searching for binding sites of advanced glycation end-products. RAGE can be identified in a number of organs including the lungs, heart and skeletal muscle and are found to be expressed in endothelial cells, mononuclear phagocytes, mesangial cells and vascular smooth muscle cells [41, 43]. The gene of RAGE is located on chromosome 6 within the major histocompatibility complex (MHC) III gene and is made up of a 5' flanking region to regulate protein transcription, 11 exons and a 3' untranslated region (UTR) [44]. The gene transcribes an

messenger RNA sequence of approximately 1.4 kb which will be later translated into the mature, transmembrane protein of 404 amino acids and approximately 45 kilodaltons (kDa) in size consisting of three segments [44]. The segments are divided into the extracellular domain, the transmembrane domain and the cytoplasmic domain. Within each segment, different components of the receptor have been further identified. The extracellular domain has been found to have a signalling domain from amino acids 1-22 followed by three immunoglobulin like domains: a V-type (variable) domain and two C-type (constant) domains [44]. The variable domain is made up of amino acids 23-116 and consists of the ligand-binding domain. One of the constant domains ranges from amino acid 124-221 followed by the second from amino acids 227-317. Following the extracellular domain, a short hydrophobic sequence from amino acids 343-363 corresponds to the transmembrane domain [41, 44]. The cytoplasmic tail is highly charged and stretches from amino acids 364-404 playing a crucial role in intracellular signal transduction [40, 41, 44].

1.4.1 RAGE Isoforms

Similar to other proteins of the MHC family, a number of different isoforms of RAGE have been identified. Due to this, some studies have reported differing amino acid sequences for the protein. The various forms of RAGE allow it to play a diverse role within the human body. There are some isoforms that have been well identified and characterized but some are not well understood. Aside from the native full length form, some of the more common isoforms that have been identified include dominant negative RAGE (dnRAGE), N-truncated RAGE (NtRAGE), endogenous secretory RAGE (esRAGE) and soluble RAGE (sRAGE) [37]. These isoforms of RAGE can play different effects in the body and further study is needed to determine which variants are physiologically relevant [45]. This leaves much to be studied to better

understand the generation of RAGE, its variant forms and how they affect physiological functions.

Table 2: Major isoforms of the receptor of advanced glycation end-products

Membrane Bound Isoforms	Characteristic	Function
Full length RAGE	Full length, 45 kDa transmembrane protein	Binds to ligands and is a transducer of intracellular signalling upon activation
Dominant negative RAGE (dnRAGE)	Lacks variable region of extracellular domain	Is unable to bind to ligands (fails to activate intracellular signals)
N-truncated RAGE (NtRAGE)	Lacks cytosolic tail	Can bind ligands but cannot trigger signalling due to lack of cytoplasmic domain
Circulating Isoforms	Characteristic	Function
Endogenous secretory RAGE (esRAGE)	A splice variant that does not contain the transmembrane domain	Binds to extracellular ligands and acts as a decoy molecule
Soluble RAGE (sRAGE)	Is generated by proteolytic cleavage of the full length receptor by metalloproteinases	Binds to ligands and acts as a decoy molecule to suppress activation of the full length receptor

1.4.2 Receptor Activation

RAGE is activated upon the ligation of its ligands to the V-type domain found at the cell surface [44]. The receptor is fairly promiscuous and has the capability to recognize and bind a variety of ligands. Through analysis, the receptor-ligand interaction was established and it was found that the receptor recognized different three dimensional structures such as beta sheets and fibrils [40]. The receptor can bind ligands including β-amyloid peptides, amyloid A peptides, AGEs and members of the S100/calgranulin and high mobility group box-1 (HMGB1) family [44].

1.4.3 Signalling Cascade & Downstream Pathways

Upon activation of the receptor in the extracellular space by one of its ligands, the signal is transduced down a protein cascade. The activation of RAGE and its signalling pathway is not yet fully understood as ligation of its ligands can trigger a number of different cascades. Ligation of an appropriate ligand to the receptor leads to phosphorylation of RAGE by protein kinase C (PKC) zeta at the serine 391 site [46]. There is no specific signalling motif that has been associated with receptor activation [46]. Triggering the receptor leads to a downstream protein cascade pathway which involves the interaction of many different proteins including phosphotidylinositol 3-kinase (PI3K), mitogen-activated protein (MAP) kinases, janus kinase and signal transducer and activator of transcription (Jak-STAT) as well as proteins in the Rho family of GTPases such as cell division control protein (Cdc) 42 and Ras-related C3 botulinum toxin substrate (Rac) 1 [47]. Sakaguchi et al. [46], has identified the ability for the cytoplasmic region of RAGE to interact with the adapter proteins, toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) and myeloid differentiation primary response gene 88 (MyD88). Signalling of RAGE through TIRAP and later through MyD88 is a key interaction that potentiates the activation of nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB), a well-established pathway in inflammation. Inhibition of the TIRAP/MyD88 signal displayed a reduction in well known pathologies displayed upon normal RAGE activation such as induction of interleukin (IL) 6 and apoptosis in endothelial cells [46].

RAGE interaction and signalling can also occur through an intermediate protein known as diaphanous-1 (mDia1) [48]. The mDia1 FH1 domain binds to the cytoplasmic domain of RAGE and triggers some of the downstream effects that are observed. There is uncertainty whether this particular interaction is responsible for all further signals although it has been

established to be responsible for eliciting the response by Cdc42 and Rac1, necessary for cellular migration [44, 46].

With the activation of these signalling cascades, a number of effects can be seen ranging from increased oxidative stress to cell survival. It is important to note that the activation of these pathways have been implicated in the development and potentiation of disease states.

1.5 Inflammation in Diabetes

Inflammation is a physiological response historically associated with redness, pain, heat and swelling but the concept has been further developed and the cellular processes have become better understood [49–51]. The inflammatory response is a defense mechanism in response to tissues that experience an insult leading to the recruitment and localization of blood cells, and other cellular factors triggered by chemokines and cytokines to the area of damage (Table 3). There are a large variety of mediators that can play a role in the inflammatory pathway.

Table 3: Inflammatory cytokines implicated in development of diabetes mellitus

Type of Diabetes	Cytokines [52–54]
Type I	IL-1 TNFα Aberrant production of interferon (IFN)-γ
Type II	IL-1β (primarily) TNFα

1.5.1 Implications in Diabetes

The term "metaflammation" has been coined to describe metabolically induced inflammation [4]. The immune response to metabolic dysfunction was first identified in *Drosophila* and is believed to benefit the organism by allowing for the redistribution of nutrients

and mediating metabolism [4, 55]. The synchrony between metabolism and immune system in these organisms permit a healthy equilibrium to be reached. Unfortunately, humans have not yet evolved this mechanism of immune system regulated metabolism. As a result, the overload of nutrients and sedentary lifestyle has led to the obesity epidemic.

In the context of type I and II DM, inflammatory factors are involved in the development and progression of disease. Type I DM, characterized by ß islet cell destruction is linked to an autoimmune inflammatory response [56]. The initial activation of pattern recognition receptors leads to early inflammation of islet cells which are further amplified once chemokine and cytokine production is active [56]. Persistent cytokine signalling has been associated with the prolonged islet cell death incapacitating the individual from sufficient insulin production. Comparatively, type II DM as a condition of metabolic dysfunction has been tied to factors such as abnormal cytokine production, acute phase reactants and activation of inflammatory signalling pathways [55]. In the early 1990s, a study by Hotamisligil and colleagues [57] described the up-regulation of a key inflammatory factor, tumour necrosis factor (TNF) α in obesity. This observation tied together the role of inflammation in obesity and diabetes. Many studies have found the increase in TNF-α and other cytokine factors such as IL-6 to be linked to insulin resistance, a main condition of type II DM [57, 58].

1.5.2 AGEs & NF-κB

One of the key issues that have been identified in diabetes as a result of the increase in AGEs is the activation of NF-κB. This particular transcription factor is a major player in the activation of many inflammatory pathways. Studies have shown by electrophoretic mobility shift assays that AGE specific ligation to RAGE can lead to NF-κB translocation from the cytoplasm

into the nucleus by the phosphorylation, dissociation and eventual degradation of the inhibitor of κB (IκB) [59–61]. Translocation of NF-κB to the nucleus permits it to bind to DNA allowing for the transcription of certain cytokines, pro-thrombotic factors and adhesion molecules. Some anti-apoptotic proteins are also under the control of NF-κB [60].

The activation of NF-κB resulting from the accumulation of AGEs is of concern due to its self-potentiating ability. Signalling through RAGE leads to the *de novo* synthesis of NF-κB as a result of the cell's production of IκB to replenish the degraded products. Unfortunately, the cell is unable to maintain sufficient levels of IκB in the cytoplasm to disrupt the translocation of newly synthesized NF-κB to the nucleus. This results in an accumulation of excess NF-κB in the nucleus amplifying the inflammatory signals [40]. Further, the deleterious effects caused by AGEs can also be observed in the up-regulation of RAGE to the cell surface. It has been found that RAGE-dependent activation of NF-κB increases the generation of the receptor which also contributes to intensifying the inflammatory response [61].

1.6 Oxidative Stress

The concept of oxidation is well known in chemistry and its relation to free radical production is widely discussed in association with pathological states. It is important to understand the implications of oxygen and nitrogen derived free radicals in physiological conditions and how they may affect the health of an individual. A disruption in the balance between the generation of free radicals and anti-oxidants can play a role in the development of disease [62]. The imbalance between oxidants and anti-oxidants with a preference for pro-oxidants leads to the damaging condition of oxidative stress. Since its introduction, many clinical presentations are thought to be associated with the generation of reactive oxygen and nitrogen

species (ROS/RNS) [63]. Some of the more extensively studied diseases associated with a dysregulation in oxidant-anti-oxidant equilibrium include cancer, diabetes, atherosclerosis and some neurogenerative diseases [64–67].

1.6.1 Role in Inflammation

The broad response during inflammation can in part lead to the development of oxidative stress. During the inflammatory process, the cellular response through the recruitment of leukocytes and mast cells can lead to an increase in oxygen consumption by the mitochondria and ultimately an increase in ROS production [67, 68]. Simultaneously, the up-regulation of ROS can act as a trigger to propagate the inflammatory pathway through factors such as NF-κB. The presence of ROS not only alters the inflammatory response but also contributes to the generation of AGEs.

1.6.2 Oxidative Stress in Diabetes

As previously mentioned, the generation of ROS has been implicated in the pathogenesis of DM. A study by Giardino et al. [69], identified the role of hyperglycemia in the formation of intracellular ROS in bovine aortic endothelial cells. Similarly, Yu et al. [70], identified this effect in neonatal rat cardiomyocytes and the embryonic rat myocyte cell line H9c2, with those sustained in high glucose conditions displaying two times greater levels of intracellular ROS accumulation compared to those maintained in normal culture conditions. Consequently, they postulated that the imbalance of reduced and oxidated states can lead to oxidative stress but does not necessarily result in cell death in all affected cells. It is believed that each cell responds to its

environment differently and the cell's fate is dependent on the levels of ROS and the cell's ability to respond to the increase in ROS [70].

There is supporting evidence that the increase in ROS generation due to hyperglycemia is sourced largely from the mitochondria. A significant amount of ROS is produced in the electron transport chain as a result of electrons escaping the process and reacting with oxygen to generate free radicals and later ROS [38, 70]. ROS generation has been linked to increases in AGE formation with manganese superoxide being a primary initiator of this event [71]. The mitochondrial proteins, manganese superoxide dismutase (MnSOD) and uncoupling protein-1 (UCP-1), play a role in regulating ROS production. Overexpression studies of MnSOD and UCP-1 under conditions of hyperglycemia have shown protective effects against the up-regulation of ROS and other pathogenic outcomes such as cell death and collagen synthesis [38, 72]. A study on adipocytes has shown that AGEs can play a role in diabetic complications as they diminish cell sensitivity to insulin through ROS up-regulation [73].

It is of no surprise that administration of anti-oxidants and free radical scavengers is protective against the increase in ROS production found in DM [69]. In addition to protecting from ROS, Giardino et al. [69], also reported decreases in AGE formation as well.

1.7 Oxytocin

Oxytocin is a peptide hormone that is most commonly associated with parturition due to its uterotonic activity [74]. It is a neurohypophysial nonapeptide first discovered by Dale in 1906 and is primarily synthesized in the paraventricular and supraoptic magnocellular nuclei of the hypothalamus [75]. Oxytocin is first synthesized as a prepropeptide in conjunction with its carrier protein neurophysin [74]. Following synthesis, oxytocin is moved by axonal flow during

which it is subject to cleavage and modifications until it arrives in the neurohypophysis and stored inside neurosecretory granules [75]. The mature, active form of oxytocin consists of nine amino acids with a disulfide bridge between cysteine 1 and 6 [75].

Initial studies of oxytocin by Dale and later Ott and Scott found that oxytocin had two primary effects, uterine contractions and milk ejection [76, 77]. In the early 1960s, Pickford described other potential physiological effects of oxytocin outside the realm of the uterus, particularly on water equilibrium [75]. A few years later, De Wied made observations between oxytocin and other central effects where administration of oxytocin caused changes in behaviour [75]. It is becoming increasingly apparent that oxytocin plays a multifaceted role in human physiology and an important role in the heart.

Oxytocin has a striking resemblance to another neurohypophysial peptide, arginine vasopressin (AVP). These two genes are both found on the same chromosome but transcribed in opposite directions. Although structurally similar to AVP, they differ by two amino acids in the 3rd and 8th position with oxytocin containing neutral amino acids and AVP containing basic amino acids (Figure 1) [74, 78]. This slight difference in sequence causes the two peptides to play physiologically different roles in the human body. Oxytocin has been found to be associated with natriuresis, vasodilatation, decreased blood pressure, negative inotropic and chronotropic effects as well as decreased cortisol levels [79–81]. AVP acts in an opposite manner to oxytocin triggering effects such as vasoconstriction, water retention and corticotropin release [79].

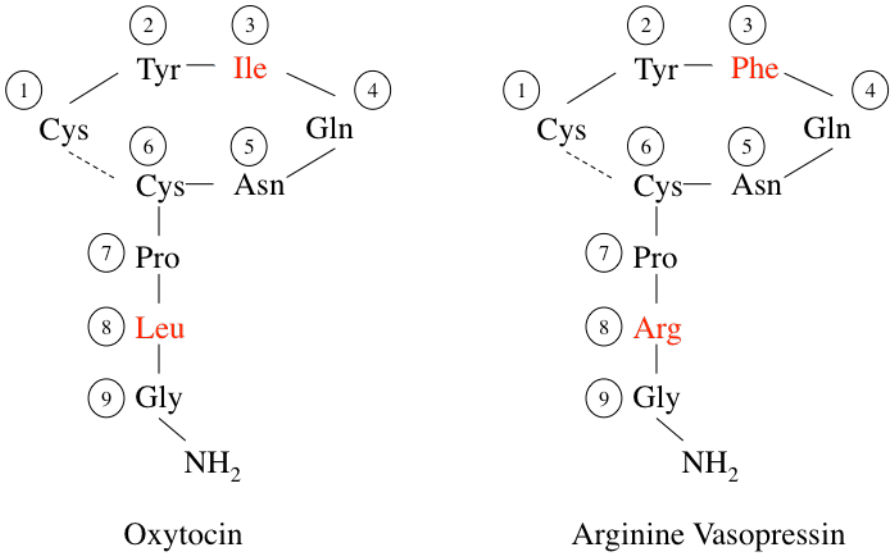


Figure 1: Structure of oxytocin and arginine vasopressin. Adapted from [78].

1.7.1 Oxytocin Receptor & Action of Oxytocin

Upon stimulation, the peptide is released from the posterior pituitary into the blood stream. The release of oxytocin can occur in a pulsatile or a continuous fashion depending on the nature of the stimulus [75]. When released, oxytocin is able to bind to its corresponding oxytocin receptor (OTR), which belongs to a class I family of G-protein coupled receptors (GPCR). As with other GPCRs, OTR is made up of the characteristic seven transmembrane α-helix domain. Binding of the ligand to the receptor triggers receptor activation and the generation of inositol triphosphate (IP) and 1,2 diacylglycerol (DAG) [74]. The production of IP and DAG leads to the release of intracellular calcium (Ca^{2+}) stores and stimulation of PKC further leading to phosphorylation of protein targets respectively. The release of intracellular Ca^{2+} activates downstream pathways including but not limited to endothelial nitric oxide synthase (eNOS), soluble guanylyl cyclase, natriuretic peptides, as well as the MAP kinase, extracellular-signal regulated kinase (ERK) 1/2 [82–84].

If the receptor stimulus is continuous, the receptor can become desensitized to further agonistic activity through phosphorylation and arrestin activity. During receptor desensitization, the receptor undergoes endocytosis, internalization and sequestration. At this point, the receptor can be degraded by lysosomes or recycled back to the plasma membrane. A study by Gimpl and Fahrenholz [74], found that 5 to 10 minutes following agonist stimulation, human embryonic kidney (HEK) 293 fibroblastic cells displayed OTR internalization. It was found that OTR along with ß-arrestin is internalized through claithrin coated pits [85, 86]. Once the receptor is dephosphorylated, it is recycled back to the cell surface and resensitized to activation by its ligand [85].

OTR is a promiscuous receptor that has been reported to have equal or only up to 10 times greater affinity for oxytocin over AVP [74]. In comparison, AVP has 30 times greater affinity to one of its receptor subtype, V_1R, over oxytocin [87]. Due to this effect, it is possible to observe cross-activation by both oxytocin and AVP through the vasopressin receptor family or OTR respectively.

1.7.2 Effects of Oxytocin on CVD

Although there are the classical effects traditionally associated with oxytocin administration, our lab has identified the entire oxytocin system in the human and rat heart amongst other functional roles for this peptide [82, 88]. Of particular interest are the effects of oxytocin on the cardiovascular system.

Interestingly, studies utilizing oxytocin have shown its capacity to act in a cardioprotective manner. Many researchers have identified a role for oxytocin and its receptor in the heart. The use of oxytocin in the cardiovascular system has been implicated in changes of

inotropy and chronotropy, natriuresis, vasodilation and endothelial cell hyperplasia [89, 90]. Oxytocin action is not limited to its direct effects on heart function, but it has also been found to induce protective cellular signalling by initiating anti-oxidant and anti-inflammatory mechanisms [90, 91]. Other work has established a protective role by oxytocin in conditions of ischaemia-reperfusion potentially through the recruitment of a signalosome complex (Gonzalez-Reyes et al., unpublished data). More recently, work with oxytocin has led to the discovery of its use as a myogenic agent. A study by Danalache et al. [84], demonstrated the differentiation of stem cells into cardiomyocytes induced by the use of oxytocin. These findings suggest that oxytocin can play a vital role in the development of cardiomyocytes as well as contribute to the functional capacity of the heart.

1.7.3 Effects of Oxytocin on DM

There have also been indications that oxytocin has a function in glucose regulation and a potential role in mediating blood glucose levels [92]. Although oxytocin has previously been reported to strongly stimulate insulin release, oxytocin itself has demonstrated insulin-like characteristics which make it a peptide of interest in the study of DM [93, 94]. A study by Florian and colleagues [93], showed that cardiomyocytes stimulated with oxytocin displayed greater levels of glucose uptake via the insulin signalling pathway. These results are further supported by results demonstrating a decrease in blood glucose levels in diabetic mice upon chronic administration of oxytocin (Plante et al., unpublished data).

A more recent study by Zhang et al. [95], demonstrates the metabolic effects of oxytocin and its analogs in rodents and humans. Administration of oxytocin intraventricularly prior to a glucose tolerance test resulted in both improved glucose tolerance and fasting insulin levels.

Similar results were attained when oxytocin was administered peripherally. Oxytocin not only presented anti-diabetic characteristics but when administered intranasally in humans, subjects showed indications of decreased body weight, reductions in the hip and waist circumference as well as normalization of the blood lipid profile [95]. With obesity being one of the key contributing factors to the development of type II DM, these results present the potential for oxytocin to tackle another aspect of the disease. The administration of oxytocin appears to be multi-faceted acting on a number of factors to diminish the effects of DM. Further study of this peptide for pharmacotherapy may reveal additional benefits in subject suffering from DM.

1.8 Hypothesis

On the basis of the described background information, we formulated the following hypothesis via these mechanisms:

1. Oxytocin can play an anti-inflammatory role in the cardiovascular system in the context of type I and II DM
    a. Oxytocin diminishes oxidative stress and inflammatory signals triggered by RAGE signalling
2. The anti-inflammatory effect of oxytocin is in part caused by oxytocin's effect on NF-κB
3. Under conditions of DM, oxytocin provides a protective effect by two mechanisms, directly through glucose regulation and indirectly through cytoprotective mechanisms affecting RAGE and NF-κB

1.9 Research Aims

The following aims have been proposed:

1. To establish the anti-inflammatory effects of oxytocin on *in vivo* models of type I and II DM
2. To study the cellular mechanisms behind oxytocin treatments in an *in vitro* model of cardiomyocytes under conditions of elevated AGEs
3. To establish the relation between RAGE signalling and NF-κB in DM

2. Materials & Methods

2.1 Animal Models

2.1.1 Streptozotocin-induced Diabetic Rats (Type I Diabetes)

Administration of STZ in adult Wistar rats causes pancreatic swelling and eventually degeneration of Langerhans β-islet cells and induces experimental diabetes mellitus within two to four days [21]. In this study, the male Wistar rats weighing 200-250 g were obtained from Charles River and treated with a bolus dose of STZ (65 mg/kg, Sigma-Aldrich, S0130) intravenously for the induction of type I diabetes. Two days following injection, blood glucose readings were taken and diabetes was indicated by blood glucose readings that were higher than 20.0 mmol/L. Rats were randomly assigned to oxytocin treatment (125 ng/kg/h) or vehicle. Treatments began in four-week-old rats for six weeks via subcutaneously implanted osmotic pumps (Alzet, 2006). Rats were housed in standard temperature conditions in 12h/12h light/dark controlled rooms. The animals had water and standard rodent diet available *ad libitum*.

2.1.2 *db*/*db* Diabetic Mice (Type II Diabetes)

Male, diabetic prone, C57BL/KsJ-leptdb-leptdb strain (*db*/*db*) mice were obtained from Jackson Laboratories with lean, non-diabetic (*db*/+) littermates used as control animals. Mice were randomly assigned to receive chronic oxytocin treatment (125 ng/kg/hr) or saline by subcutaneously implanted osmotic pumps. Treatments began in four-week-old mice and continued for 12 weeks. After six weeks, the osmotic pumps were replaced to adjust oxytocin concentration to the body weight of the mice. The mice were housed in standard temperatures in a controlled room allowing for 12h/12h light and dark cycles with water and standard rodent diet available *ad libitum*. The body weight of the animals were measured prior to the beginning of the

experiments and twice weekly during the protocol. An oral glucose tolerance test (OGTT) was performed on the 16-week-old mice to evaluate the effect of oxytocin on glucose tolerance.

2.1.3 Picrosirius Red Staining of Aorta

Paraffin sections were dewaxed and rehydrated by placing slides in xylene for five minutes twice and into decreasing ethanol solutions (95%, 75%, 50%) for five minutes each and finally into distilled water. Nuclei were stained using Weigert's haematoxylin for eight minutes and washed in running tap water for 10 minutes. Slides were then stained with aqueous Picrosirius red (Sirius red in picric acid) for one hour and then washed with acidified water (0.5% (v/v) glacial acetic acid) two times. Excess water was shaken off and slides were dehydrated in increasing concentrations of ethanol (50%, 75%, 95%) for five minutes each and finally in xylene. Samples were mounted on Permount and dried overnight. Slides were imaged using an inverted microscope (Olympus IX51) and captured by a digital charge coupled camera (QImaging)

2.1.4 Periodic Acid Schiff Staining of Aorta

Samples were prepared and stained for periodic acid Schiff by the pathology department of the Centre Hospitalier de Universite de Montreal.

2.1.5 Western Blot of Left Ventricle

Left ventricular tissues were quickly snap frozen and stored at -80ºC until processing. Tissue was prepared for immunoblotting using cell lysis buffer 10X (Cell Signal, 9803) with added 5mM sodium fluoride (Santa Cruz, sc-24988) and protease inhibitors (Sigma Aldrich,

P2714). Cells were then homogenized and vortexed for 30 minutes prior to extensive sonication. Cells were centrifuged at 12000 rpm for 20 minutes and cell supernatant isolated from the pellet.

Proteins concentrations were then measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, PI23227) through the enhanced microplate protocol. Isolated supernatants were prepared in two dilutions and quantified through the use of a 5-250 μg/mL bovine serum albumin (BSA) standard curve. Preparations were loaded into clear, flat bottom, 96 well plates and read using a spectrophotometer at 562 nm.

Tissue supernatants were then prepared for loading by the addition of distilled water and Laemmli buffer 5X (pH 6.2, 300 mM Tris HCl, 50% (v/v) glycerol, 10% (m/v) sodium dodecyl sulphate (SDS), 5% (v/v) ß-mercaptoethanol, 0.05% bromophenol blue) to generate uniform concentrations for all samples. Samples were then heated up to 95ºC for five minutes.

SDS-polyacrylamide separation gels (29% (m/v) acrylamide/1% (m/v) bis-acrylamide) were prepared at a concentration of 10% (m/v) at 1.0 mm or 1.5 mm thickness depending on the protein to be detected with 10% stacking gels. Equal quantities of prepared whole cell lysates (5-15 μg) were loaded alongside a protein standard (Bio-Rad 161-0375). Gel electrophoresis was performed using electrophoresis buffer (pH 8.3, 192 mM glycine, 20 mM Tris, 0.1% (m/v) SDS) in a Mini Protean Tetra Cell (BioRad, 165-8001) at 100-150 V/4ºC for one to two hours until the dye front was flushed out of the gel.

Prior to transfer, both gels and membrane were equilibrated in transfer buffer (pH 8.3, 125 mM Tris, 120 mM glycine, 20% (v/v) methanol) with agitation for 15 minutes. Protein transfer was carried out in a Trans-Blot Cell (BioRad, 170-3946) using 0.45 μm nitrocellulose membrane (BioRad, 162-0115) and blotting paper (VWR, 28298-020) at 30-60 V/room temperature for one hour.

All following procedures took place with gentle agitation. After protein transfer, visualization of transfer efficiency onto the membrane was performed using aqueous Ponceau red (0.2% (m/v) Ponceau Red, 0.75% (m/v) trichloroacetic acid). Acrylamide gels were stained with Coomassie blue (Coomassie brilliant blue, 50% (v/v) methanol, 10% (v/v) glacial acetic acid) and decolorized using a decolorant solution (10% (v/v) glacial acetic acid, 20% (v/v) methanol).

Membranes were quickly rinsed with distilled water and Tris-buffered saline with Tween (TBS-T, pH 7.4, 20 mM Tris, 150 mM sodium chloride, 0.1% (v/v) Tween-20). Blocking of non-specific sites was done with 5% (m/v) BSA for phosphorylated proteins or 5% non-fat dry milk in TBS-T for one hour at room temperature. Membranes were briefly rinsed with TBS-T once prior to the addition of primary antibodies (RAGE, phospho-NF-κB & total NF-κB obtained from Abcam and Cell Signal) which were prepared in a solution of 2% (m/v) BSA in TBS-T with 0.05% (m/v) sodium azide (Sigma Aldrich, S2002) and incubated overnight at 4ºC or for one hour at room temperature. Membranes were washed with TBS-T for five minutes three times and then incubated with horseradish-peroxidase (HRP) conjugated secondary antibodies prepared in 5% (m/v) skim milk blocking solution for one hour at room temperature.

Proteins expressed at low levels were detected by an alternative method. Following application of the primary antibody, membranes were incubated with biotinylated forms of the antibody against the animal in which the primary was raised diluted in TBS-T with 5% (m/v) non-fat dry milk blocking solution for 30 minutes. Membranes were rinsed twice for five minutes with TBS-T and then incubated with streptavidin-HRP for 30 minutes.

Detection of signal for all proteins was completed using enhanced chemiluminescence (GE Healthcare Life Science, RPN 2106/2135/2232) and visualized by autoradiography.

Analysis of data was performed by densitometry measurements using ImageJ (NIH, Maryland) and normalized to a standard housekeeping protein (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or ß-actin) or total proteins in the case of phosphorylated proteins.

2.1.6 Reactive Oxygen Species Accumulation

Reactive oxygen species accumulation was observed by performing immunohistochemistry on frozen sections of the heart obtained from both animal models. The experiment was performed using the OxyIHC Oxidative Stress Detection Kit (Millipore, S7450) as per the manufacturer's recommendations. In short, cryosections were fixed to slides using methcarn fixative (60% (v/v) methanol, 30% (v/v) chloroform, 10% (v/v) glacial acetic acid) overnight at 4ºC. Following fixation, antigen retrieval was performed by submergence in heated sodium citrate buffer for five minutes and allowing the slides to cool at room temperature for 30 minutes. Slides were washed using 1X wash buffer three times for five minutes. Slides were blotted dry and a Super PAP Pen (Daido Sangyo, 22309) was used to create a hydrophobic barrier around the tissue. All following incubations were performed at room temperature with five minute washes (three times) between each step except for after the addition of primary antibody. 2,4-dinitrophenylhydrazine (DNPH) solution was added to the slides and incubated in a dark, humidified chamber for 30 minutes. The sample was then blocked using 1X blocking buffer for 30 minutes and freshly prepared primary antibody was added for one hour. Biotinylated secondary antibody was added for 30 minutes. To saturate endogenous peroxidases, samples were incubated with 3% H_2O_2 for 10 minutes. Streptavidin-conjugated HRP was then added for 30 minutes followed by a fresh preparation of 3,3'-diaminobenzidine (DAB) for five minutes. Samples were rinsed with 1X wash buffer to stop colour development. Haematoxylin

was added for five minutes and development stopped by rinsing the slide in warm running tap water. Slides were then dehydrated by submerging in multiple baths of alcohol of increasing concentration (70%, 90% and 100%) and finally in xylene. Slides were mounted using Permount (Fisher Scientific, SP15-500) allowed to dry and visualized using an inverted light microscope.

2.1.6 TUNEL Assay of Cellular Apoptosis

Detection of cellular apoptosis was performed using the DeadEnd Fluorometric TUNEL system (Promega, TB235) according to the manufacturer's instructions. Briefly, left ventricular tissue was cryosectioned and fixed with TissuFix (Chaptec) for 15 minutes at room temperature. Samples were washed in PBS for five minutes two times at room temperature. Following this, samples were permeabilized by incubation in a solution of proteinase K (20 μg/mL) for eight to ten minutes and then washed by submersion in PBS for five minutes. Samples were fixed once again in TissuFix for five minutes and washed with PBS. Equilibration buffer was added at room temperature for five to ten minutes followed by labelling with rTdT reaction mix for one hour at 37ºC in a humidified chamber protected from light. Slides were submerged in saline sodium citrate solution for 15 minutes to stop the reaction and washed in PBS three times. Slides were mounted and fixed using Prolong Gold antifade reagent with DAPI (Invitrogen, P36931), allowed to dry and viewed through an inverted microscope.

2.1.7 Study Approval

All studies were performed according to the guidelines of the Canadian Council of Animal Care (CCAC) and performed under protocols approved by the Centre de Recherche du Centre Hospitalier du Universite de Montreal Ethics Committee.

2.2 Cell Culture

2.2.1 H9c2 Cell Line

Commercially available H9c2 (2-1) embryonically derived rat cardiac myocytes were obtained from the ATCC. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate (ATCC, 30-2002) and further supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, 260140-079) and 1% (v/v) penicillin/streptomycin (Gibco, 15140-122). Cells were maintained in an incubator at 37ºC and 5% CO_2 (Sanyo MCO-17AC). Cells were grown until 70-80% confluency to maintain the expected phenotype.

For most experiments, the cells were seeded at a density of 6000 cells/cm^2 and allowed to attach overnight. Following seeding, cells were starved with reduced serum, 1% (v/v) FBS, overnight to allow for cell cycle synchronization.

2.2.2 *In vitro* glyceraldehyde-derived AGE Generation

In vitro production of glyceraldehyde-derived advanced glycation end-products was completed as previously described by Takeuchi et al. [96]. In brief, 0.1 M glyceraldehyde (Sigma, G5001) was combined with 50 mg/mL BSA in a 0.2 M phosphate buffer solution (pH 7.4). A corresponding control solution containing 50 mg/mL BSA and no glyceraldehyde was prepared in parallel. Solutions were syringe filtered through a 0.22 μm filter, placed in a ventilated T25 cell culture flask (Sarstedt, 83.1810.002) and stored in a humidified 37ºC, 5% CO_2 incubator for seven days. Following incubation, the solution was purified by diafiltration against phosphate buffered saline (PBS) using Amicon Ultra filters (EMD Millipore, UFC903008). Excess glyceraldehyde was washed out through this process by centrifugation at

3000 g for 25 minutes per cycle for a total of five cycles. The concentrate was then resuspended in the initial volume of PBS.

2.2.2.1 AGE Activity Verification

Previous reports have indicated superior fluorescent activity of glycated proteins in comparison to BSA solutions at the 370/440 nm excitation and emission spectrum respectively [97]. In order to verify the *in vitro* AGE product, a serial dilution of the glyceraldehyde-AGE and BSA was performed and plated into clear, 96 well plates. The plate was then read using the Synergy 2 Microplate Reader (Bio-Tek) at the 370 nm excitation and 440 nm emission spectrum.

2.2.2.2 AGE Product Stability Assay

To confirm the stability of the AGE products, an experiment similar to the activity verification were performed. Serial dilutions of AGE and BSA controls were prepared in PBS and loaded into clear, 96 well, flat bottom plates. The plate was read at 370 nm excitation and 440 nm emission over a period of 24 hours to confirm its ability to maintain fluorescent activity following multiple freeze-thaw cycles.

2.2.3 PrestoBlue Evaluation of Cell Metabolism

Cells were seeded in black walled, 96 well plates (Nunc 165305) and allowed to attach overnight. Cells were starved with DMEM supplemented with 1% FBS overnight to synchronize cell cycles. Treatment of the cells took place on the following day. Cells were treated with glyceraldehyde-AGE or its corresponding control in both a time-dependent and dose-dependent manner. Following treatment, PrestoBlue (Life Technologies, A-13261) was added to each well

and incubated for up to 2 hours at 37ºC/5% CO_2. The plate was then read using a fluorescent plate reader set at excitation 535±20 nm, emission 590±20 nm.

2.2.4 In Cell ELISA

Cells were plated in clear, 96 well plates (Corning, 3595) and allowed to attach overnight. Cells were then starved overnight in DMEM supplemented with 1% FBS the following day. Cells were treated with glyceraldehyde-AGE or the corresponding BSA control in a time and dose dependent fashion. Following treatment, cells were fixed in TissuFix diluted in PBS (1 in 4) for 20 minutes at room temperature or overnight at 4ºC.

The plate was extensively washed using wash buffer (0.1% (v/v) Triton X-100 (Pharmacia Biotech, 171-315-01) in PBS) five times. Cells were then permeabilized using permeabilization buffer (0.5% (v/v) Triton X-100 in PBS) for 15 minutes. Cells were washed with wash buffer five times. Blocking of non-specific sites was performed with blocking buffer (1% BSA (m/v) in PBS supplemented with 10 mM glycine and 0.2% (m/v) sodium azide) for 30 minutes at room temperature. Primary antibody was prepared in blocking buffer at the appropriate concentrations and incubated overnight at 4ºC. The plate was then washed five times with PBS-Tween 20 (0.1% (v/v) Tween-20 in PBS). Biotinylated secondary antibody at the appropriate dilution (1:10000-1:20000) was prepared in blocking buffer and incubated at room temperature for 30 minutes. No secondary negative controls were also prepared. Cells were quickly washed with PBS-Tween five times and then incubated in streptavidin-conjugated HRP diluted (1:10000) in PBS for 30 minutes at room temperature. Cells were washed extensively with PBS-Tween three times followed by PBS twice. 1X TMB ELISA Substrate Solution (eBioscience, 00-4201-56) was added and the signal was allowed to develop from 5-30 minutes.

The plate was then read using a spectrophotometer set at 630 nm. To normalize for cell number, Janus Green (0.3% (m/v) Janus Green (Spectrum, JA110) in distilled water) whole cell stain was added to each well and incubated for five minutes at room temperature. Extensive washing with distilled water was performed to wash out excess dye. 0.5 M hydrochloric acid was added to each well and the plate was read using a spectrophotometer at 595 nm.

2.3 Statistical Analysis

All results are presented as the mean ± standard error of mean. Data was processed using Prism 6 (Graphpad Software, California). One-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test were performed when specified. Grouped data was analyzed by two-way ANOVA with a Bonferroni post-hoc test. Results were considered to be significant when $p<0.05$.

3. Results

3.1 *In vivo* Results

3.1.1 Metabolic effects of oxytocin

3.1.1.1 Effects of oxytocin on body weight

Before experiments were performed, there were no differences in body weight observed in the rats selected to the STZ or control group as well as in the *db*/+ and *db*/*db* mouse groups. Figure 2 demonstrates the effects of oxytocin on animal body weight following chronic oxytocin treatment. The STZ rats receiving vehicle treatment showed a significant decrease in weight (401.2 ± 6.55 g) compared to control (551.9 ± 21.60 g) (Fig. 2A). Those animals treated with oxytocin displayed a tendency towards an increase in body weight (443.7 ± 23.95 g).

Comparatively, in the model of type II diabetes (Fig. 2B), the *db*/*db-veh* group of animals (52.3 ± 1.45 g) were significantly more obese when matched to their *db*/+ control counterparts (26.4 ± 0.44 g). Oxytocin treatment of *db*/*db* mice for a period of 12 weeks resulted in a very significant decrease in body weight (44.9 ±1.86 g). The ability for oxytocin to reverse obesity in type II DM may indicate the direct effect of oxytocin on certain metabolic functions.

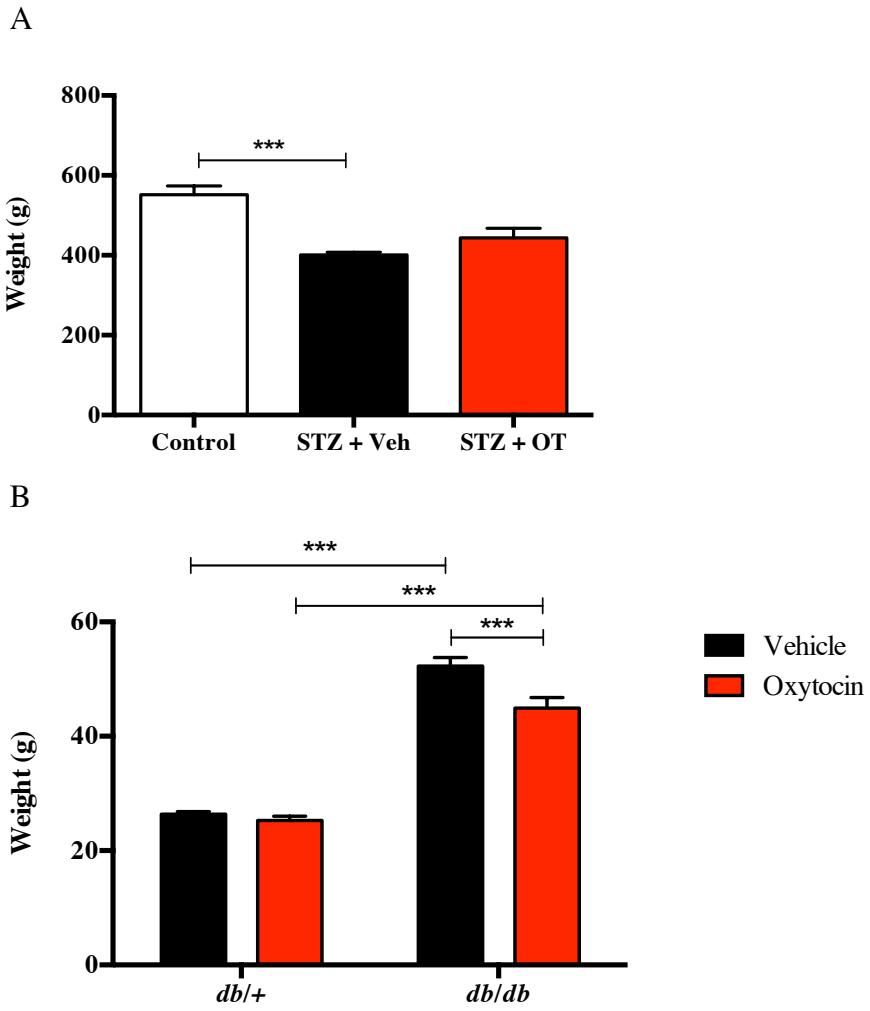


Figure 2: Body weight of control and diabetic animals. A) Control, vehicle treated and oxytocin treated rats and B) *db*/+ and *db*/*db* control and oxytocin treated mice had their body weights measured upon conclusion of the study (***p<0.0001).

3.1.1.2 Glycemic management with oxytocin

To assess the function of oxytocin on glucose plasma level, control and STZ rats were treated with a single dose of oxytocin. The animals had blood glucose levels monitored immediately prior to injection with oxytocin and at 1 hour following administration. In control animals (Fig. 3A), we observed that oxytocin increased blood glucose levels in the plasma. Although this change was seen in control animals, the glucose levels were still considered to be in the normal, non-diabetic range. In contrast, the diabetic STZ rats (Fig. 3B) responded to oxytocin treatment with a significant decrease in blood glucose concentration. These results support previous reports that oxytocin has the capacity to trigger glucose uptake *in vivo* as well as in isolated myocyte cultures from skeletal muscles and the heart [92, 93, 98]. The insulin-like effect of oxytocin in this context may further highlight its pleiotropic actions.

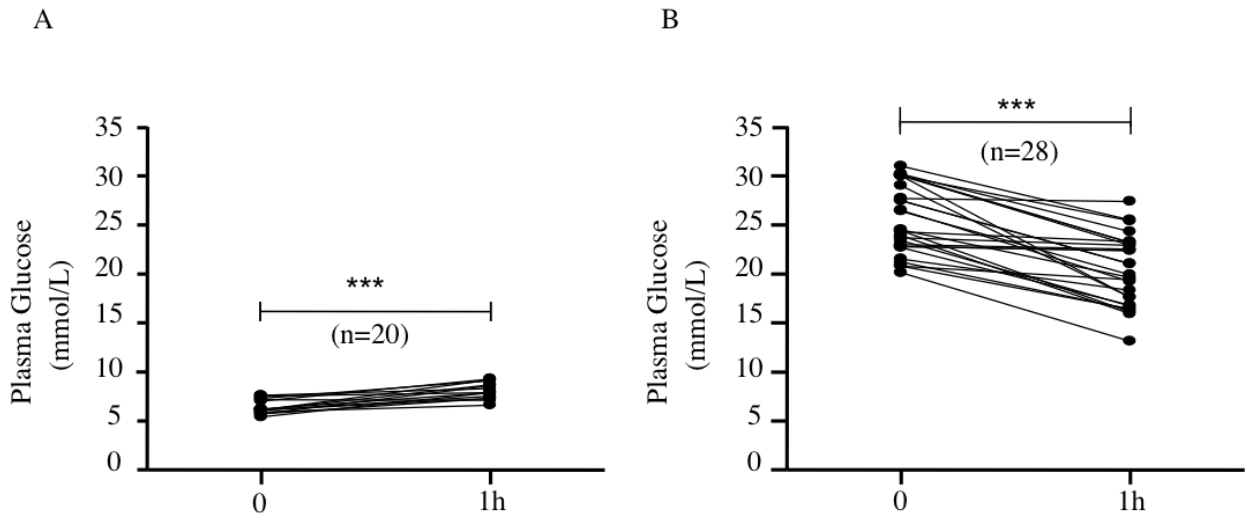


Figure 3: Glycemic management of blood glucose levels. A) Control and B) STZ induced diabetic rats had a bolus dose of oxytocin administered and their plasma glucose levels measured at baseline and 1 hour following treatment (***p<0.0001).

In addition to the immediate effect of oxytocin administered as a single bolus dose, it was also critical to establish its chronic effects on plasma glucose levels. To evaluate this, an OGTT was performed in both animal models of diabetes. Animals treated with STZ (Fig. 4A) presented slightly higher baseline blood glucose levels compared to the oxytocin treated group. Following administration of glucose, it becomes apparent that rats treated with oxytocin demonstrated a faster return of glucose plasma concentration to initial levels compared to those receiving vehicle. The vehicle treated group of STZ rats displayed elevated blood glucose levels even two hours after glucose supplementation.

The mice of *db*/+ genotype (Fig. 4B) displayed a normal blood glucose profile upon glucose administration regardless of whether they received chronic oxytocin treatment or not. We further observed that *db*/*db* animals receiving vehicle had higher blood glucose levels when subjected to an OGTT compared to that of those given oxytocin (Fig. 4C). *Db*/*db* mice treated with oxytocin had significantly lower blood glucose levels throughout the two hour period. This analysis indicates the improvement of glucose tolerance upon chronic oxytocin administration in two diabetic animal models. It appears that oxytocin can act in both an acute and chronic fashion as well as induce insulin-like effects for the long term management of DM.

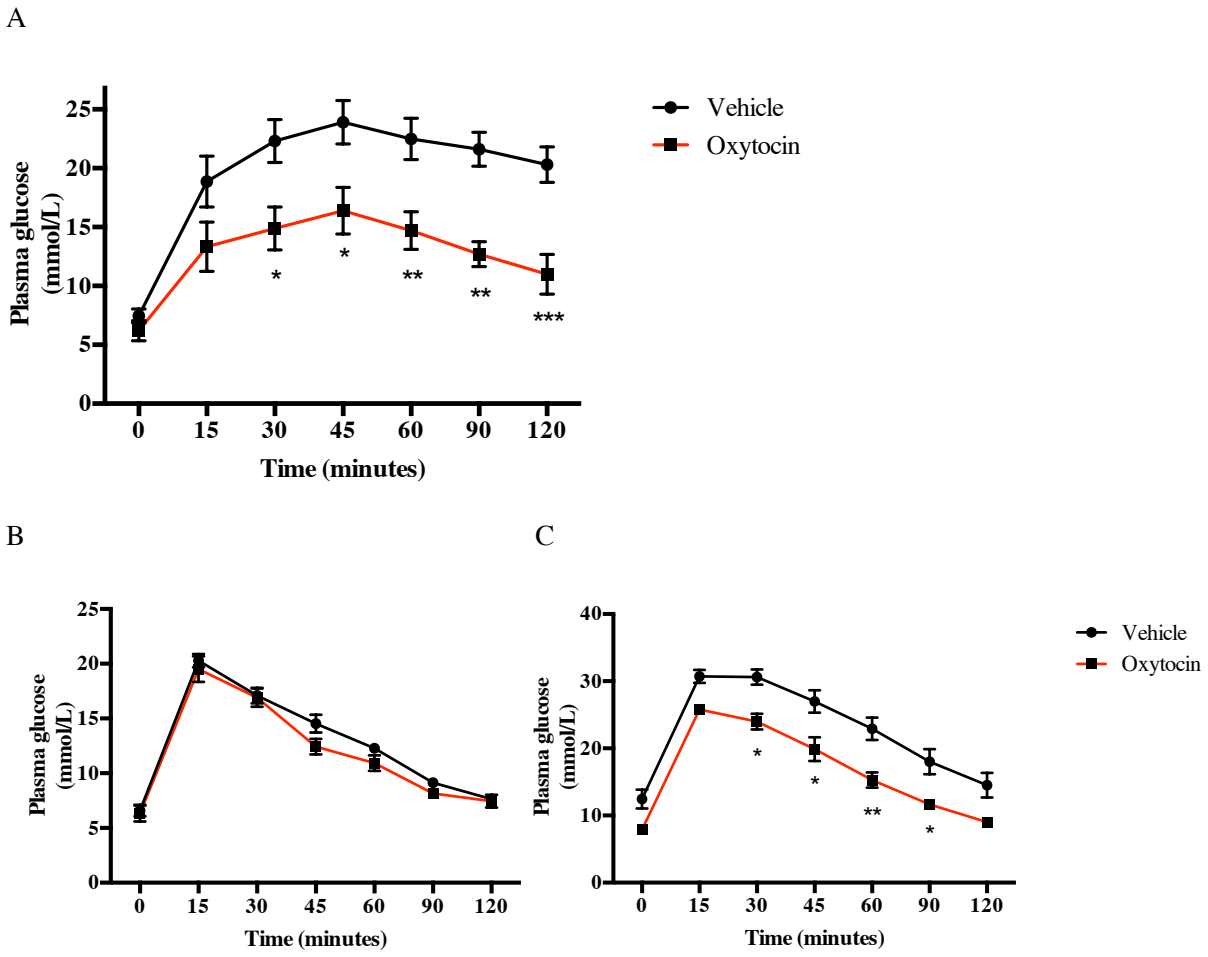


Figure 4: Effect of oxytocin treatment on oral glucose tolerance test in diabetic models. Blood glucose levels of diabetic animals were measured in 15 minute intervals for 120 minutes in STZ rats and *db*/*db* mice following 6 week chronic oxytocin treatment. A) Oral glucose tolerance test of STZ diabetic rats which have been treated with vehicle or oxytocin. Plasma glucose of B) *db*/+ and C) *db*/*db* mice (*p<0.05, **p<0.01, ***p<0.0001).

3.1.2 Evaluation of Cardiac Weight

The structural changes in the heart can lead to abnormalities in cardiac function. Since DM is directly linked to the development of cardiovascular diseases, we determined the role of oxytocin treatment on cardiac morphology using heart weight as a basic parameter (Fig. 5) contributing to cardiac pathology. In Figure 5A, we observed that there was no noticeable change in vehicle treated STZ rats when normalized to their body weights compared to control animals (1.013 ± 0.03 fold vs. 0.968 ± 0.03 fold in control animals). In STZ rats that were treated with oxytocin, there was also no significant change compared to control animals (1.022 ± 0.03 fold).

When observing the hearts of *db*/*db* mice representing type II diabetes, there was a very significant decrease in the heart to body weight ratio compared to the normal *db*/+ animals (0.591 ± 0.01 fold vs 1.000 ± 0.02 fold in *db*/+). Upon treatment with oxytocin in the *db*/+ group there was no effect on the heart to body weight ratio (1.053 ± 0.03 fold) compared to the vehicle treated group. Similarly, in the *db*/*db* group of mice, we did not observe any positive changes in heart weight in the oxytocin treated group (0.610 ± 0.02 fold) compared to the vehicle group.

In addition to assessing for potential structural changes of the heart, the aorta in STZ-treated diabetic animals was stained to determine if the vasculature was affected as well. In histological sections stained with periodic-acid Schiff (Supplementary Figure 1), control rats had no accumulation of polysaccharides at the aorta whereas those which were diabetic had a significant polysaccharide deposits. Animals that had received chronic oxytocin treatment demonstrated a regression in polysaccharide accumulation compared to the diabetic animals. Similar results were obtained when the aorta was stained with picrosirius red for collagen detection (Supplementary Figure 2).

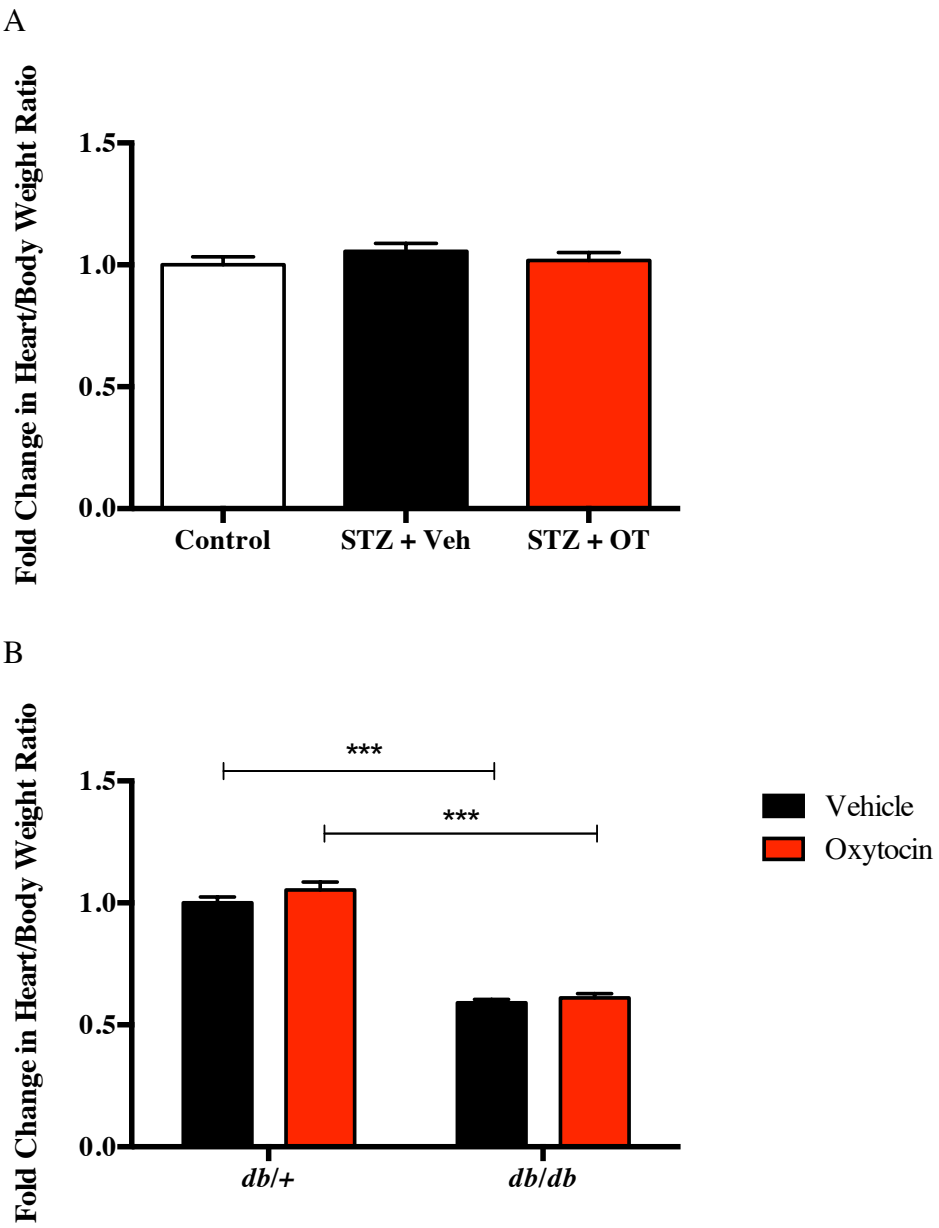


Figure 5: Heart weight of STZ and *db*/*db* animals. Total heart weight was measured in both A) STZ rats and B) *db*/*db* mice and normalized to the body weight of each animal. Results were then expressed as a fold change compared to the control and *db*/+ vehicle treated animals respectively. (***p<0.001).

3.1.3 DM modifies protein expression of RAGE and downstream pro-inflammatory factors

In order to further elucidate the mechanism behind cardiovascular pathology, we investigated RAGE to determine if there were any changes in protein expression that could link the development of CVD to DM. As a result, we noticed that both models of DM had significantly elevated levels of the RAGE protein in the left ventricle of the heart.

Using Western blot analysis (Fig. 6A), we have found in vehicle-treated STZ rats, RAGE protein levels being 3.56 ± 1.01 fold higher in comparison to the normal rats (1.00 ± 0.17) and oxytocin treated STZ rats (1.10 ± 0.40). Correspondingly, in *db*/*db* mice (Fig. 6B), RAGE protein expression in control (1.00 ± 0.03) animals were significantly lower than those seen in the *db*/*db* mice receiving vehicle (1.55 ± 0.05). The cardiac RAGE level in *db*/*db* mice receiving oxytocin was in the range of normal levels (1.13 ± 0.01). The results obtained from STZ rats and *db*/*db* mice receiving oxytocin provide substantial evidence that oxytocin treatment is capable of reducing RAGE expression in the heart. This data suggests that the RAGE signal transduction blockade by oxytocin may be an important strategy for the prevention of the deleterious cardiovascular consequences of diabetes.

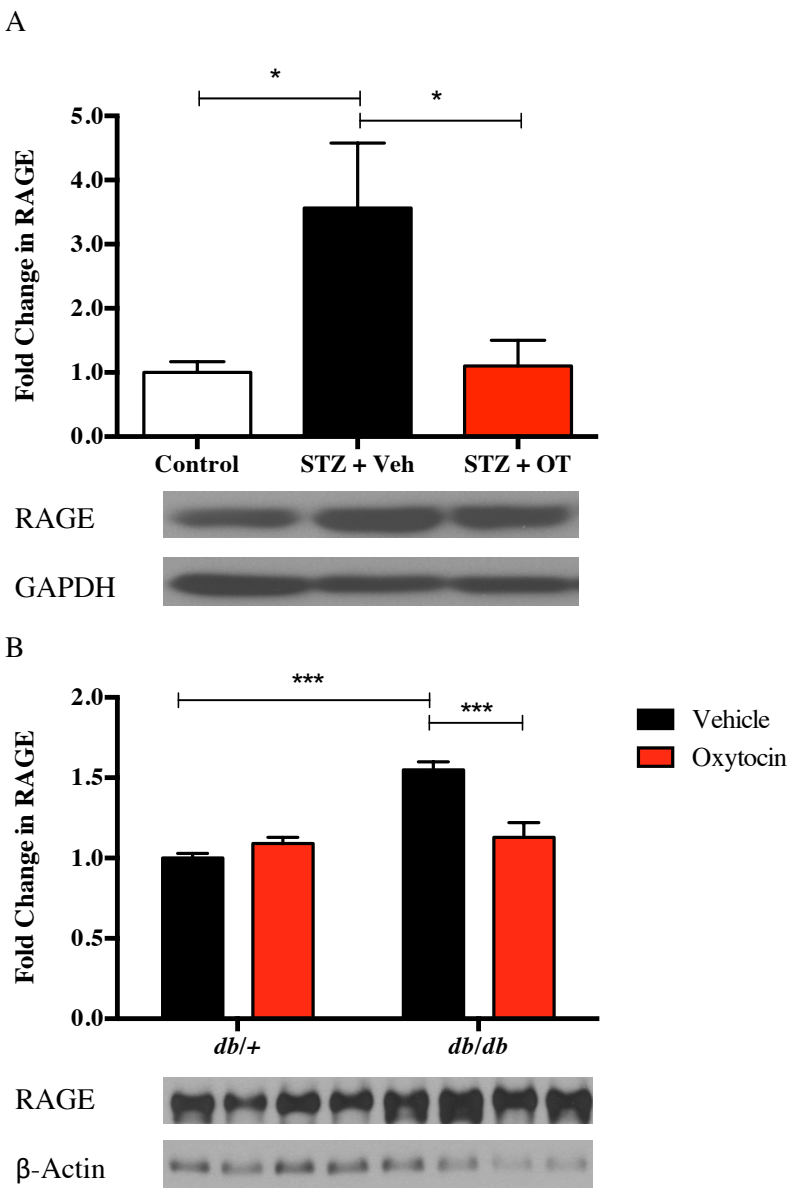


Figure 6: RAGE expression in the left ventricle of diabetic animals. Protein extracted from the left ventricle of diabetic animals was measured for changes in RAGE expression. A) Fold change of RAGE in control, vehicle and oxytocin treated animals. Data was normalized to GAPDH and fold change is expressed relative to the control animal group. B) Changes in RAGE expression in *db*/+ and *db*/*db* animals that have been treated with vehicle or oxytocin. Data was normalized to β-actin. Fold change is expressed relative to the *db*/+ vehicle group (*$p<0.05$; ***$p<0.0001$).

Through RAGE signalling, it has been observed that NF-κB activation is accentuated and propagated [47], therefore it is of interest to determine the effect of experimental diabetes on the phosphorylation and expression of NF-κB in the heart. In this study, no significant changes in either phosphorylated (Fig. 7A) (0.87 ± 0.04 fold vs. 1.00 ± 0.09 fold in control) or total form of NF-κB (Fig. 7C) (0.93 ± 0.06 fold vs. 1.03 ± 0.16 fold in control) was seen in the hearts of vehicle treated animals. Additionally, STZ animals that were chronically infused with oxytocin did not alter either phosphorylated (1.00 ± 0.12 fold) or total (1.01 ± 0.15 fold) levels of NF-κB in the heart.

In the *db*/*db* mouse model, the level of phosphorylated NF-κB (Fig. 7B) was not significantly different in the hearts of *db*/+ and *db*/*db* models whether they were infused with oxytocin or not. Interestingly, when total NF-κB was studied (Fig. 7D), our results indicated that the *db*/*db* animals (1.22 ± 0.06 fold) had significantly higher levels of NF-κB compared to their *db*/+ counterparts (1.00 ± 0.05 fold). The *db*/*db* mice that were treated with oxytocin, displayed a significant reduction in NF-κB (0.90 ± 0.06 fold). These results suggest that the inflammatory effects of NF-κB may be more pronounced in type II DM compared to that of type I DM.

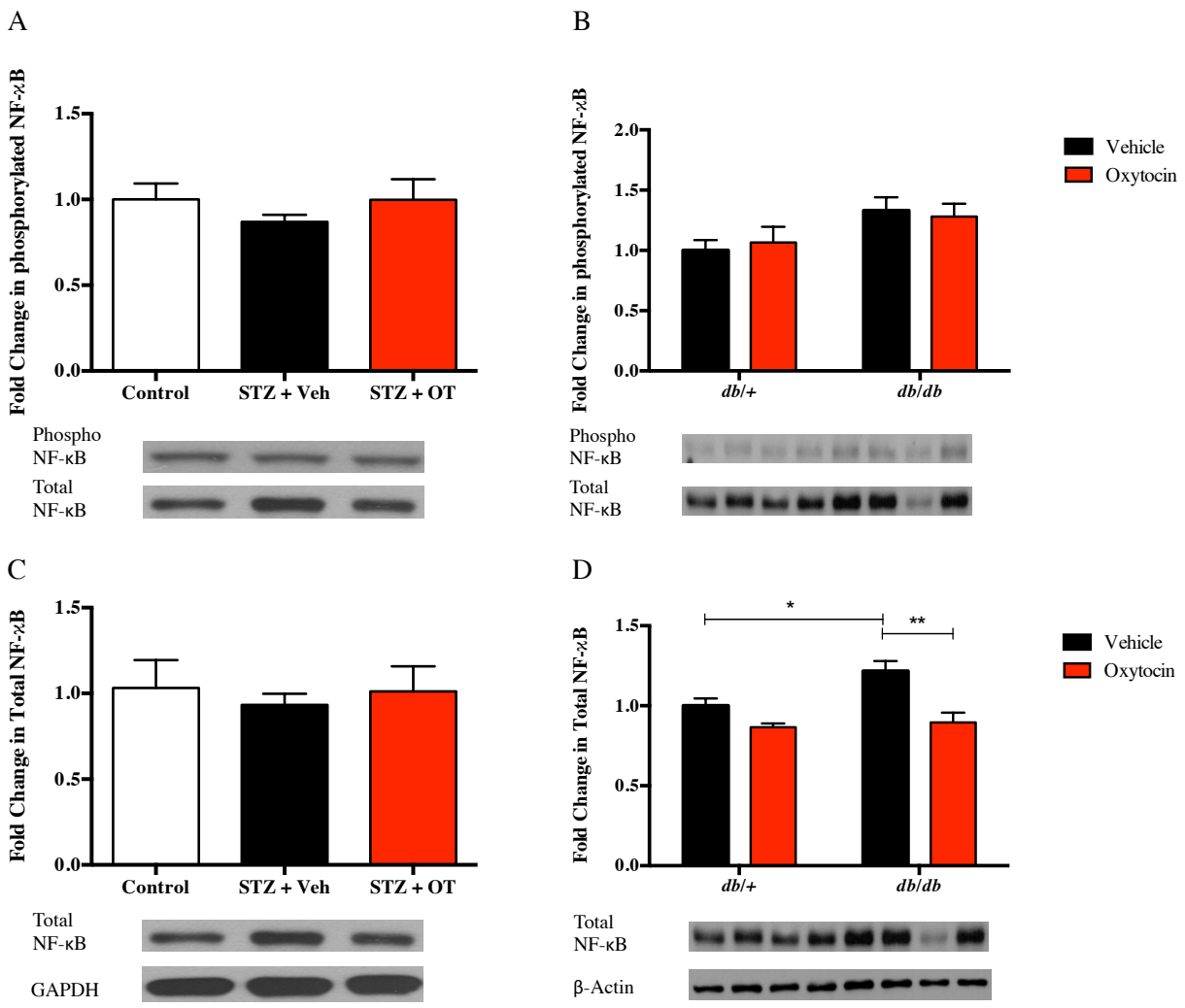


Figure 7: Protein expression of phosphorylated NF-κB and total NF-κB in the left ventricle of diabetic animals. Left ventricular tissue from STZ and *db*/*db* animals were used for protein extraction and measured for changes in the phosphorylated and total forms of NF-κB. A) Protein level changes of phosphorylated NF-κB expression in control, vehicle and oxytocin treated animals and in B) *db*/+ and *db*/*db* animals treated with vehicle or oxytocin. Data was normalized to total NF-κB and fold change is expressed relative to the control animal group. Total NF-κB protein expression was measured in C) STZ animals and in D) *db*/*db* animals. Data was normalized to β-actin and fold change is expressed relative to the control or *db*/+ vehicle treated group (*$p<0.05$, **$p<0.01$).

3.1.4 DM may amplify the accumulation of reactive oxygen species in left ventricular tissue

In the condition of DM, a key cause of concern to the cardiovascular system is the generation of ROS that may cause damage to the heart as well as the surrounding vasculature. We performed immunohistological staining of the left ventricular sections with the OxyIHC Oxidative Stress Detection Kit to determine if ROS accumulation was associated with development of cardiac alteration in both models of DM. In doing so, we noted that the normal rat (Fig. 8A) had minimal or no accumulation of ROS in the left ventricle. In contrast, the STZ rats receiving vehicle displayed the abundant accumulation of ROS in cardiac tissue outlined by brown staining and by signs of morphological alterations in cell structure (Fig. 8B). Chronic oxytocin administration of STZ rats (Fig. 8C) did not appear to improve the outcome with respect to ROS accumulation.

In the heart of *db*/+ animals receiving either vehicle (Fig. 8D) or oxytocin (Fig. 8E) treatment, the ROS staining was barely visible. The *db*/*db* animals treated with vehicle (Fig. 8F) demonstrated noticeably elevated ROS accumulation throughout the left ventricle. In *db*/*db* mice that had received oxytocin (Fig. 8G), we noted significant reductions of ROS in the heart. These results support the results of cardiac NF-κB expression suggesting that oxytocin plays a role in inhibition of cardiac inflammation and oxidative stress in the model of type II diabetes but not in those serving as a model of type I diabetes.

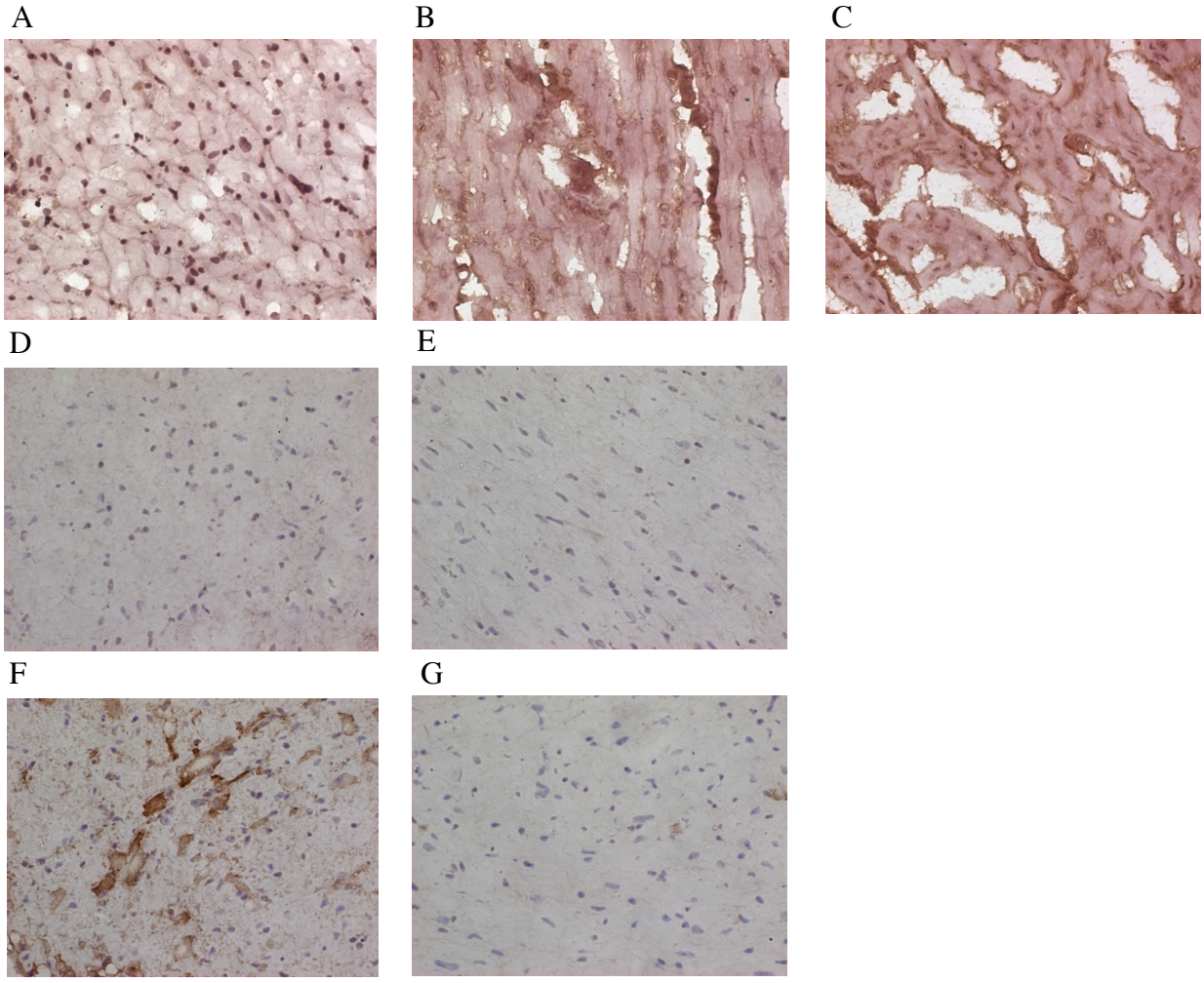


Figure 8: Immunohistochemical staining of reactive oxygen species accumulation in left ventricular tissue of type I and type II diabetics animals. Cryosectioned left ventricular tissue obtained from sacrificed animals were stained by immunohistochemistry for the accumulation of reactive oxygen species. Reactive oxygen species is stained by DAB (brown) and cell nuclei by haematoxylin (blue). A) Control, non-diabetic B) STZ diabetic, vehicle treated C) STZ diabetic, oxytocin treated D) *db*/+, vehicle treated E) *db*/+, oxytocin treated F) *db*/*db*, vehicle treated G) *db*/*db*, oxytocin treated. Images were viewed at 200X through an inverted light microscope.

3.1.5 Apoptosis of cardiac cells in diabetic animal models

In addition to metabolic abnormalities, subcellular defects, abnormal expression of proteins and other factors, the AGE-dependent signalling through the RAGE receptor evoking inflammation and ROS may contribute to the development of cardiac cell apoptosis. We used a terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay to detect DNA fragments resulting from the apoptotic cascade. In the left ventricular sections of control (Fig. 9A) and STZ rats (Fig. 9B & 9C), apoptotic cells were observed. Oxytocin treatment (Fig. 9C), of STZ rats only minimally reduced the number of apoptotic cells in cardiac sections. This result corroborates with other data obtained in this study that indicates that oxytocin treatment has no major effect on ROS accumulation in the heart of STZ rats.

TUNEL assay analysis of cardiac sections from *db*/+ mice (vehicle or oxytocin treated) (Fig. 9D & 9E), did not present with apoptotic cells (n=3 with an average of 51 cells in the field of view). In vehicle treated *db*/*db* mice (Fig. 9F) some apoptotic cells (1.43 ± 0.215% vs. 0.309 ± 0.115% in *db*/+ controls) were found in the left ventricle. Following chronic infusion of oxytocin, the left ventricle no longer displayed evidence of apoptotic cells (Fig. 9G) and are comparable to the *db*/+ control animals.

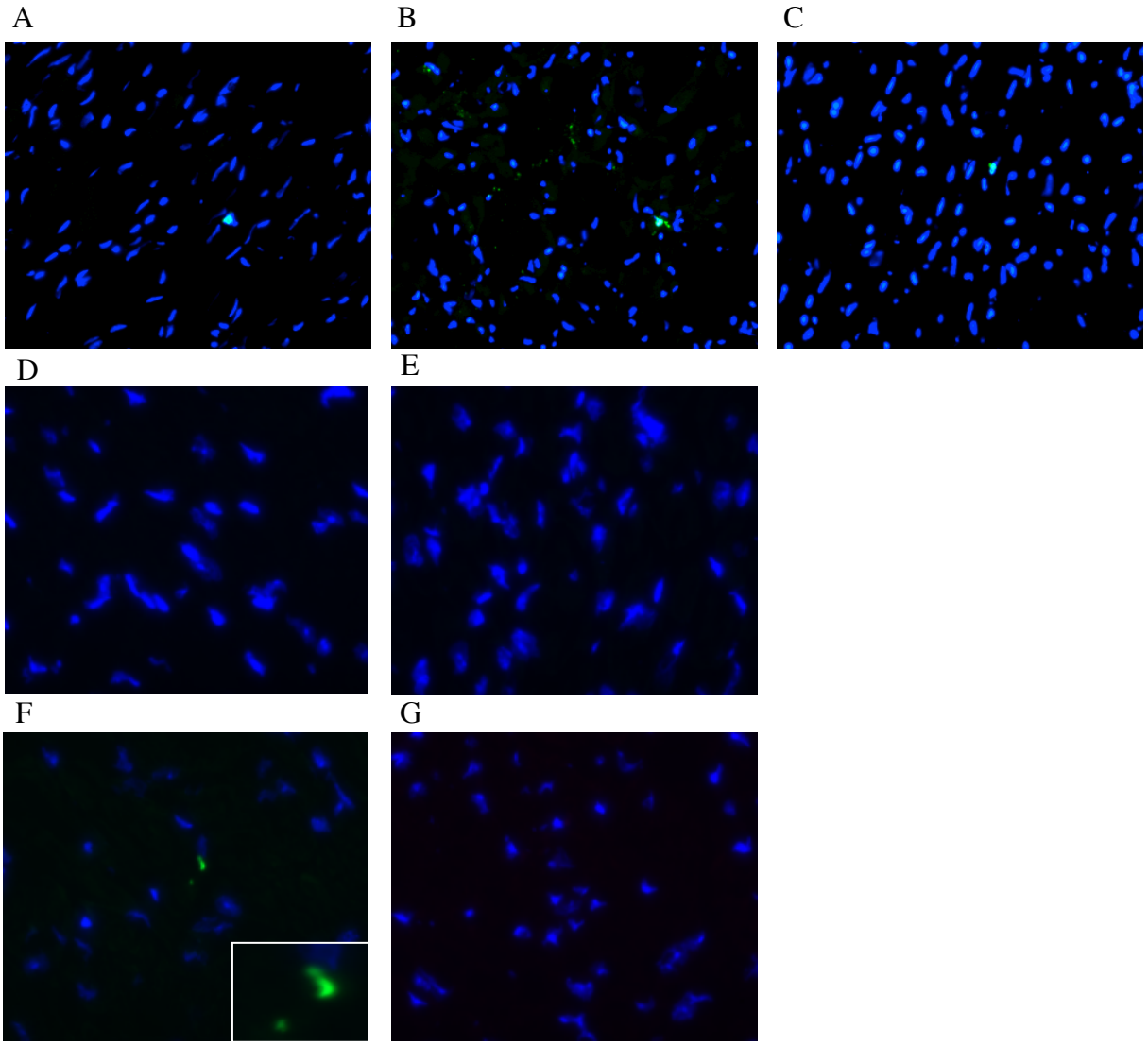


Figure 9: Cell apoptosis marked in the left ventricle of type I and II diabetic models. Apoptotic cells are marked in left ventricular tissue by fluorescent TUNEL staining. Cells presenting with apoptotic activity is stained by a fluorescent green colour and the cell nuclei is stained with DAPI (blue). A) Control, non-diabetic B) STZ diabetic, vehicle treated C) STZ diabetic, oxytocin treated D) *db*/+, vehicle treated E) *db*/+, oxytocin treated F) *db*/*db*, vehicle treated G) *db*/*db*, oxytocin treated. Images were viewed at 200X through an inverted microscope.

3.2 *In vitro* Results

3.2.1 Evaluation of *in vitro* generation of glyceraldehyde-AGE

To investigate the mechanisms of RAGE signalling in DM, we pursued an *in vitro* model of AGE stimulation. *In vitro* generation of AGEs requires the incubation of proteins such as BSA with sugars or sugar derivatives, in this case glyceraldehyde. Following incubation, and consistent with literature, we observed that BSA which had been modified displayed increased fluorescence compared to that of unglycated albumin (Figure 10) [96]. Proteins that had undergone the glycation process, demonstrate a logarithmic increase in fluorescence reaching maximum fluorescence at approximately 12.5 mg/mL of glycated protein. The curve pattern observed is consistent with fluorescence obtained in commercially generated AGEs (CellBioLabs, STA-348). Comparatively, unmodified proteins demonstrated a linear model with minimal fluorescence even at high concentrations of protein.

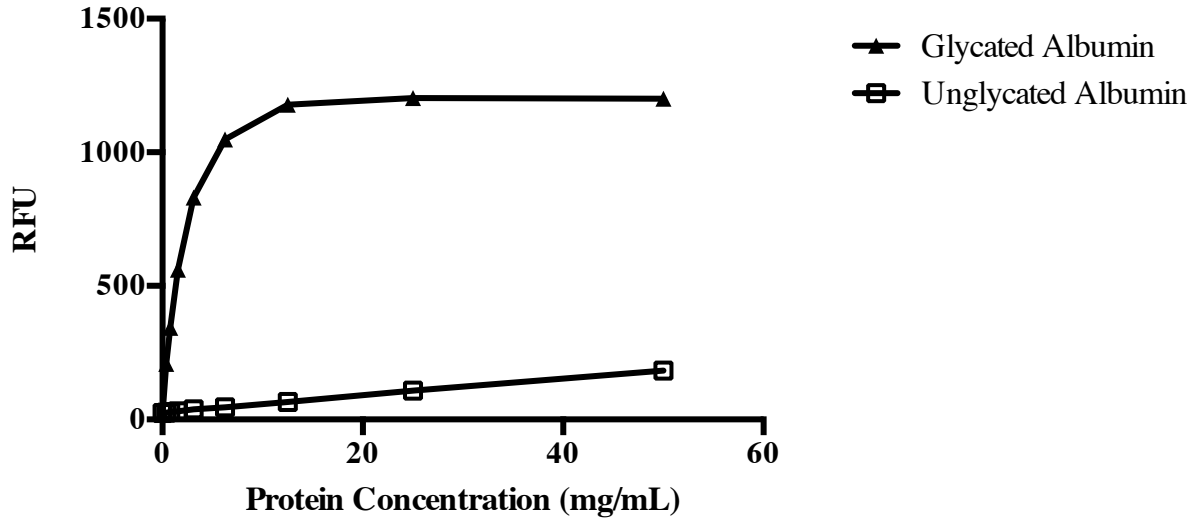


Figure 10: Fluorescence curve of *in vitro* generated glyceraldehyde-advanced glycation end-products. The fluorescence of unglycated BSA and glycated BSA following incubation with glyceraldehyde were read at various concentrations to establish the fluorescent activity found in AGEs. The curve demonstrates the fluorescent characteristic of glycated proteins compared to that of unglycated proteins. Data is plotted in relative fluorescent units (RFU).

3.2.2 Assessment of AGE product stability in a time-dependent manner

Given that it is possible for generated products to degrade over time, we studied the degradation of AGE products over the period of our desired stimulation times. Prior to stimulation of cultured H9c2 cells, we mimicked cell culture conditions and monitored the fluorescent activity of the products to note any changes. Based on the results obtained, we did not note any significant changes in fluorescent activity when time was taken into consideration (Figure 11). This sustained fluorescence from the AGEs suggests that for the duration of our experiments, we may assume that the AGEs remain stable and maintain sufficient activity to simulate the effect of elevated glycated proteins.

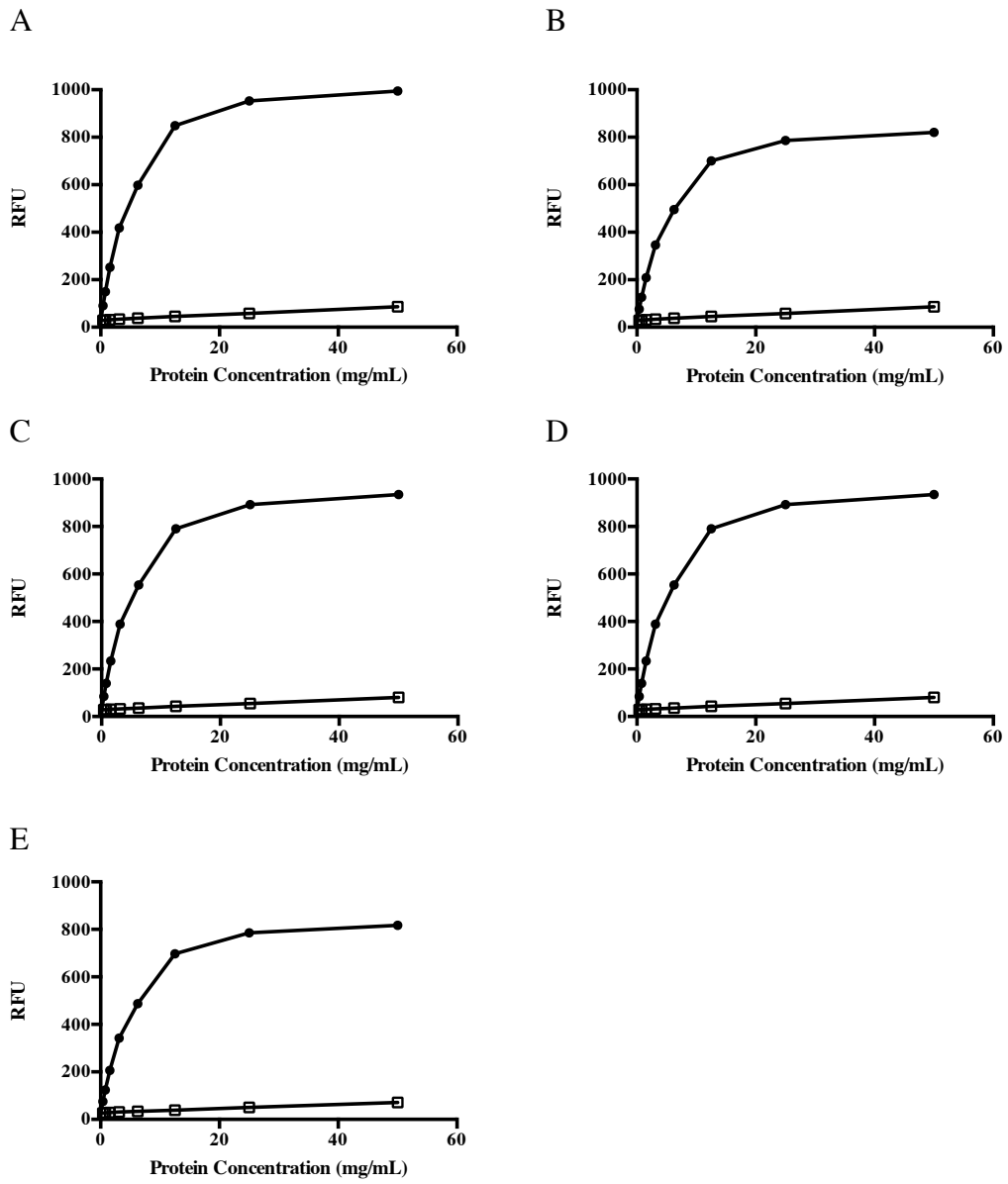


Figure 11: Assessment of *in vitro* glyceraldehyde-advanced glycation end-products stability. Activity of glyceraldehyde-AGE was assessed in a time dependent manner based on fluorescent activity. Fluorescence was measured at 430/590 nm over A) 2 hours B) 4 hours C) 6 hours D) 8 hours and E) 24 hours to determine the stability of the AGEs. AGE products appear to be stable over the 24 hour time period. Closed black circles represent glycated BSA and open white squares represent unglycated BSA. Fluorescent values are expressed in RFU.

3.2.3 Viability of H9c2 cells following AGE treatments

Having established the activity of AGEs by fluorescence, we aimed to determine both the optimal time and dose of AGEs for cell treatment without affecting cell viability. The analysis on cell viability was based on changes in cellular metabolism. In a resazurin-based assay, live cells are able to reduce resazurin to generate resorufin, a fluorescent product proportional to the number of metabolically active cells. Using this method, we determined the most appropriate dose of AGEs for cell stimulation. No significant changes in cell viability was noted at the doses ranging from 25 μg/mL to 400 μg/mL (Figure 12). Data analysis indicated increased cell metabolism compared to BSA controls at low doses (namely 25-50 μg/mL) followed by nearly equivalent effects at higher doses.

Based on these results, we designed a time-course experiment with the dose of 100 μg/mL, as the lowest dose with equivalent effect, to monitor the changes in cell viability in a culture of H9c2 cells. The cells treated with AGEs for different time periods did not show significant changes in metabolism over 24 hours (Figure 13). H9c2 cells when treated with AGEs for 1 to 8 hours revealed a slight tendency to increase cell metabolism compared to the BSA control treatment ($p=0.189$). When cells were treated for 24 hours with either BSA or glyceraldehyde-AGE, cell metabolism was not different indicating that stimulation for up to 24 hours did not involve cell toxicity.

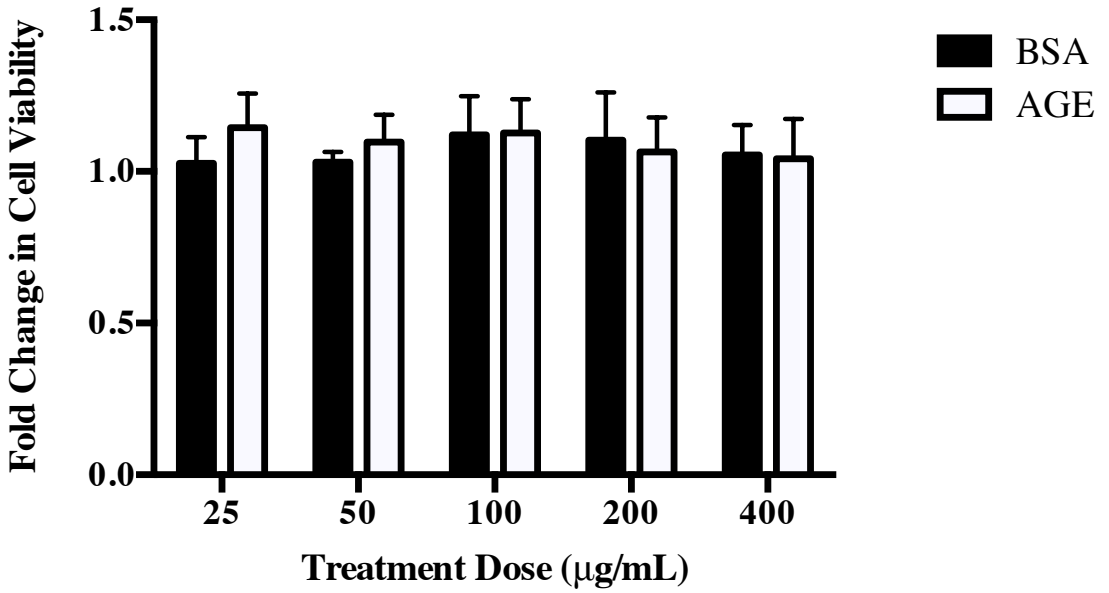


Figure 12: Dose dependent cell viability assessment of H9c2 cells treated with AGEs. H9c2 cardiomyocytes were treated with AGEs for 24 hours at various doses to evaluate the effect of AGEs on cell viability. No changes to cell viability were observed when cells were treated with increasing doses of AGEs. PrestoBlue fluorescence values were assessed using the Synergy 2 spectrophotometer at 530/590 excitation/emission. Results were normalized to cells treated with 1% FBS and analyzed using multiple comparisons between the BSA treated and AGE treated groups. Data are from three independent experiments in triplicate.

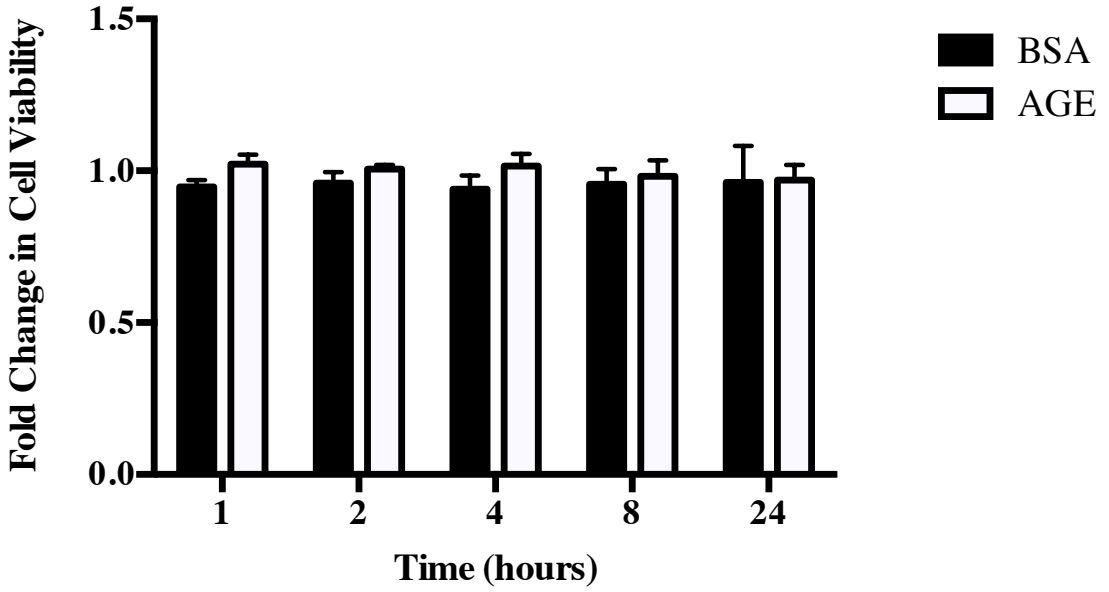


Figure 13: Cell viability test of H9c2 cells treated with AGEs in a time dependent manner. H9c2 cells were treated with 100 μg/mL AGEs in a time dependent manner to assess cell viability. Fluorescence values were assessed 530/590 excitation/emission. Results were normalized to cells treated with 1% FBS and analyzed using multiple comparisons between the BSA treated and AGE treated groups. Data are from three independent experiments in triplicate.

3.2.4 Effects of AGE stimulation on activation of NF-κB

To further explore the role of AGEs on cardiomyocytes, we investigated its effects on inflammation. It is well known that NF-κB is activated by the AGE/RAGE axis and can potentiate the inflammatory pathway. We examined the changes in protein expression of NF-κB in its phosphorylated and total form by an in-cell ELISA technique. Our results show that a time dependent stimulation with AGEs did not modify the phosphorylation of NF-κB (Fig. 14A). Similar results were observed with total NF-κB protein levels (Fig. 14B) with no significant changes compared to samples from the BSA control.

Using the same technique, we also evaluated NF-κB protein expression in response to a dose dependent AGE stimuli (Figure 15). When H9c2 cells received AGE treatment, we noted a significant increase ($p=0.0485$) in NF-κB phosphorylation (Fig. 15A) compared to those receiving only BSA. It is clear that a positive correlation ($r=0.8277$, $p=0.0836$) exists between increasing doses of AGEs and levels of phosphorylated NF-κB. On the other hand, the total level of NF-κB (Fig. 15B) calibrated to GAPDH, our housekeeping control, was not affected by AGE treatment. Instead, the protein levels of NF-κB had a tendency to remain at similar or lower levels ($p=0.1884$) than cells treated with unglycated BSA.

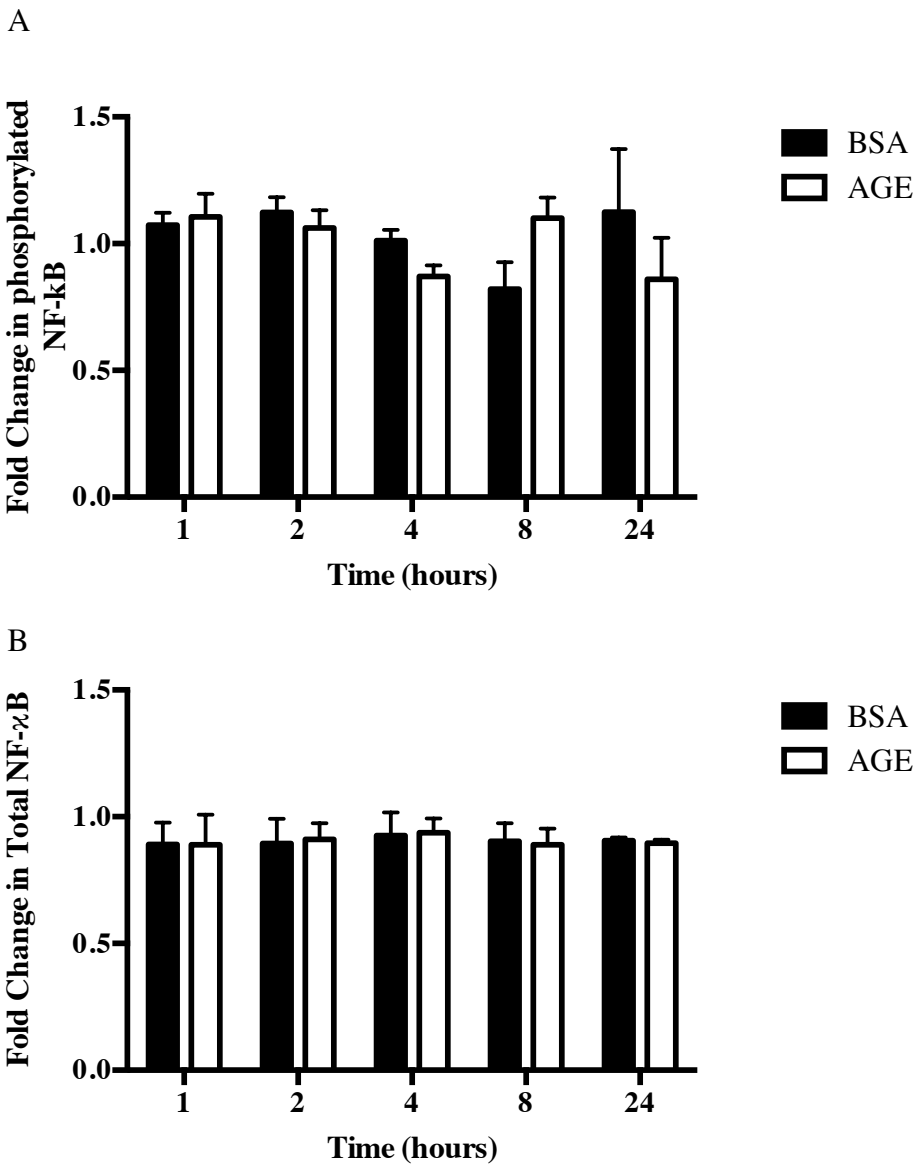


Figure 14: NF-κB protein expression through time dependent AGE stimulation of H9c2 cells. Change in levels of A) phosphorylated NF-κB normalized to total NF-κB and expressed as fold change relative to the 1% FBS control and B) total NF-κB normalized to GAPDH and expressed as fold change relative to the 1% FBS control. Data are from three independent experiments in triplicate.

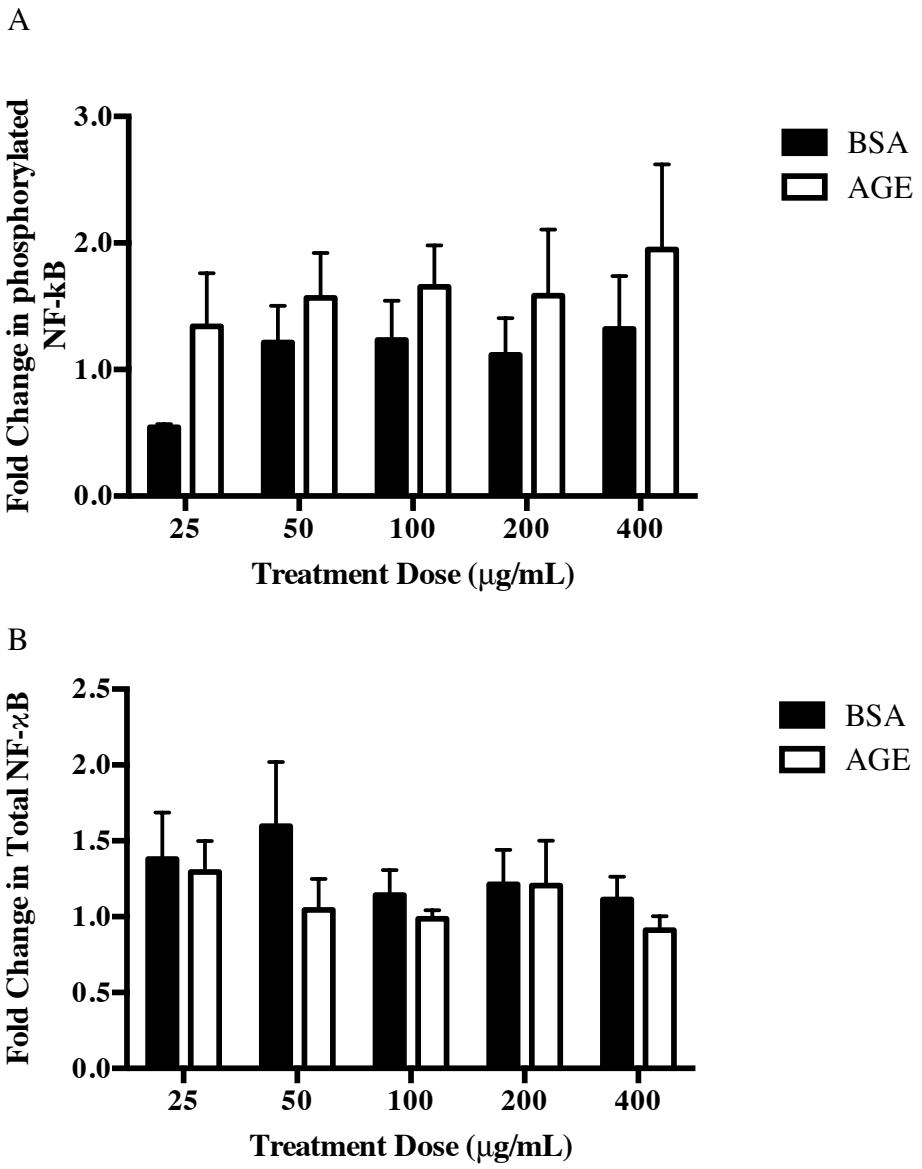
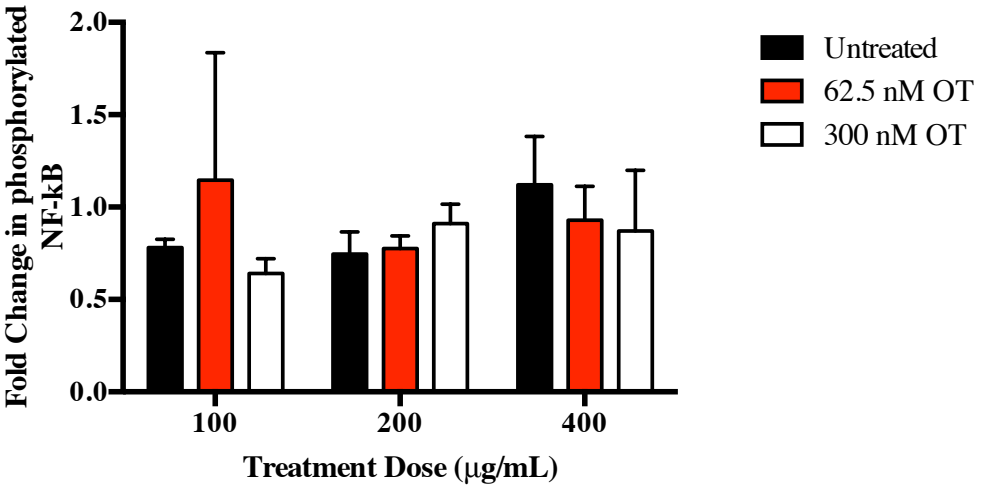


Figure 15: NF-κB protein expression through dose dependent AGE stimulation of H9c2 cells. Change in levels of A) phosphorylated NF-κB normalized to total NF-κB and expressed as fold change relative to the 1% FBS control and B) total NF-κB normalized to GAPDH and expressed as fold change relative to the 1% FBS control the FBS control. Data are from three independent experiments in triplicate.

3.2.5 Oxytocin post-treatment on NF-κB protein levels following AGE stimulation

Our lab has extensively evaluated the use of oxytocin as a therapeutic drug in the cardiovascular system. Through studies, we have demonstrated the anti-inflammatory potential of oxytocin in the rat heart subjected to myocardial infarction [99]. It is of great interest to establish the potential anti-inflammatory mechanism by which oxytocin may protect the diabetic heart. In Figure 16, the use of oxytocin was evaluated by administration at two doses: the optimal- 62.5 nM, producing protection in simulated ischemia-reperfusion experiments [100] and a high dose of 300 nM, as previously used to induce RAGE shedding in HEK 293 cells [101]. Oxytocin treatment was performed in cells treated with different AGE concentrations. The results did not show any significant changes in the phosphorylation of NF-κB (Fig. 16A) compared to that of untreated cells. This result is consistent with that observed in our *in vivo* model where level of phosphorylation is not significantly affected following oxytocin treatment. Similarly, when examining total levels of NF-κB protein expression (Fig. 16B), it is not apparent that there are significant changes ($p=0.5216$) upon treatment with oxytocin. There is also no evidence that a higher dose of oxytocin is more effective in down-regulating expression of NF-κB.

A



B

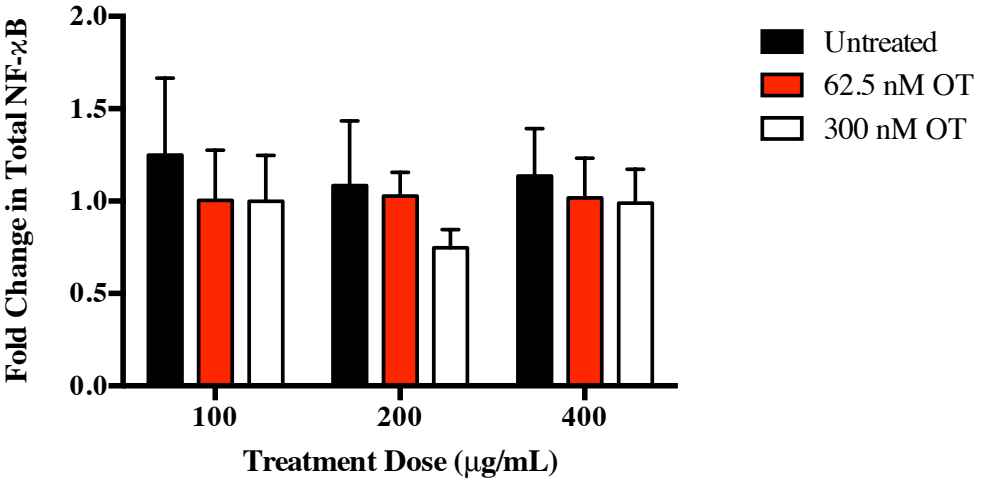


Figure 16: NF-κB protein expression following a dose dependent AGE stimulation and oxytocin treatment. H9c2 cells were pre-stimulated with AGEs followed by administration of oxytocin at two doses (62.5 nM and 300 nM). Change in levels of A) phosphorylated NF-κB normalized to total NF-κB and expressed as fold change relative to the 1% FBS control and B) total NF-κB normalized to GAPDH and expressed as fold change relative to the 1% FBS control the FBS control. Data are from three independent experiments in triplicate.

4. Discussion

Although oxytocin has traditionally held a conventional role in labour stimulation and milk letdown, the pleiotropic effects observed in cell and animal models has demonstrated its potential in many functions related to the cardiovascular system [80, 82, 93, 99, 102]. The current study further outlines the prospective mechanisms through which the positive effects of oxytocin may act with respect to cardiovascular complications seen in DM.

4.1 Oxytocin reverses metabolic and morphological changes caused by diabetes mellitus

4.1.1 Oxytocin protects against metabolic changes

A large range of metabolic effects manifest through the development of DM. Body weight is a key factor that is commonly affected by the presentation of diabetes. Consistent with other reports, body weight fluctuations are observed in both models of type I and II DM [103, 104]. In this study, the STZ animals that were treated with vehicle, demonstrated weight loss likely resulting from a loss in triglyceride stores and muscle mass [103, 105]. Although oxytocin treatment did not fully resolve the loss of body weight, the potential for weight gain further suggests that oxytocin can act in an insulin-like manner [106]. The opposite effect was observed in the *db*/*db* model of DM, which displayed a noticeable weight gain when receiving vehicle. This increase in body weight is linked to the defective leptin signalling pathway which may be a point of intervention through drug administration. Oxytocin treatment demonstrated drastic reductions in body weight of *db*/*db* animals. A previous study by Zhang et al. [107], indicates that administration of oxytocin can intervene through coordination of circadian rhythm and physiological metabolism. It is further implied that fat-enriched diets alter the rhythmic cycle of

oxytocin release making the individual susceptible to the development of obesity. Exogenous administration of oxytocin may supplement for this altered cycle of oxytocin release.

A previous study from our laboratory demonstrated that oxytocin can contribute to the management of blood glucose levels, which suggested the potential for oxytocin to decrease blood glucose levels in type I diabetes [93]. This is supported by recent results, which demonstrate improvements in glucose tolerance via decreases in baseline blood glucose levels and recovery time following glucose administration in both DM models. The oxytocin-mediated increase of glucose uptake by skeletal muscle and cardiac cells can contribute to this effect [93, 98]. It has been found that signalling through 5' adenosine monophosphate activated protein kinase (AMPK) and calcium is crucial for oxytocin mediated glucose uptake [98]. AMPK is a well-known metabolic regulator of muscle metabolism and gene expression. Administration of oxytocin may stimulate AMPK thereby providing additional protection against cardiac hypertrophy, ischemia-reperfusion injury and inflammation [100, 108].

4.1.2 Cardiac implications and vascular morphology reversal by oxytocin treatment

DM can lead to the development of associated pathologies including changes to cardiac structure. One of the greatest concerns for diabetics is the development of cardiomyopathy, later leading to heart failure. Our study has explored the effects of oxytocin on cardiac structure and morphology in both type I and II DM. It has previously been suggested by Bilim and colleagues [109], that in conditions of type I diabetes cardiac atrophy is observed. Based on our evaluation of the heart weight in STZ rats, our results did not corroborate this observation as no changes were noted when it was normalized to the animal's body weight. It is possible that that although

no morphological changes, particularly atrophy or hypertrophy, were noted, functional irregularities such as systolic and diastolic dysfunction may exist [110]. It has been shown that the metabolic processes that occur in the heart under conditions of diabetes may ultimately impair cardiac function [110]. The shift in cardiac metabolism from primarily glucose to fatty acids has been associated with the development of cardiac dysfunction through mechanisms such as modifications in calcium homeostasis, lipotoxicity and mitochondrial damage [110]. It may warrant further study in the model of type I DM to establish if changes in cardiac function can be resolved by peripheral oxytocin administration.

In this study, we were able to confirm previously reported evidence of cardiac atrophy in type II diabetic animals [111]. Administration of oxytocin in the type II diabetic animals did not elicit any significant changes in the heart weight suggesting that it may not play a role in reversion of heart morphology. As mentioned earlier, the absence of morphological change does not necessarily imply a lack of functional changes. The increases in AGE formation can lead to the deactivation of nitric oxide, an essential factor in coronary vasodilation. Development of pathology through exposure of cardiac myocytes to excess AGEs is not potentiated only through defects in NO signalling but increases in NF-κB and mitochondrial ROS formation as well as modifications in myocardial calcium handling through sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) [33, 112, 113]. Oxytocin as a well-known inducer of NO synthesis and Ca^{2+} release may correct and normalize the dysfunctional nitric oxide and calcium system correcting cardiac function [74, 98]. Oxytocin's mechanism of action may be responsible for providing cardiac protection through the triggering of natriuretic peptide and nitric oxide release [111].

Although the observations from this study did not demonstrate morphological reversion of cardiac pathology, it may indicate the possibility of resolving vascular pathology. Previously, a study by Nation et al. [114], showed that the administration of oxytocin in apolipoprotein E knockout mice would attenuate atherosclerosis. In our study, we were able to show the attenuation and reversion of both collagen and polysaccharide accumulation at the level of the aorta in STZ rats. It has been suggested that peripheral administration of oxytocin may attenuate atherosclerotic plaque formation by affecting oxidative stress and inflammation in cells localized at the vessel [114]. Although levels of ROS and inflammatory markers were not markedly changed in the heart of STZ animals within our study, further work to investigate the vasculature is needed to confirm our observations. Since atherosclerosis is an endothelial disease associated with NO dysfunction, it is important to consider the changes in vascular pathology upon oxytocin treatment as potentially being in part due to oxytocin's effect on NO synthesis. Correction of the NO system may lead to the resolution of vascular changes that are observed in diabetes [115].

4.2 RAGE is a key player in the inflammatory process found in type I and II diabetes mellitus

Our investigation on RAGE expression in animals of type I and II DM is consistent with reports from other studies. It is evident that DM, regardless of origin, amplifies the expression of RAGE. We found that no changes were observed in type I diabetic animals in both the phosphorylated or total forms of NF-κB. This suggests that although RAGE is up-regulated, there is no change in the inflammatory marker. This may be explained by a study by Mollah et al. [116], which found abnormal NF-κB function in dendritic cells and monocytes due to an

overexpression of Src homology-2 domain-containing phosphatase-1 (SHP-1) in type I diabetic patients. There is evidence that SHP-1 activity suppresses NF-κB activity as well as reduces TNF-α activation of NF-κB [117]. Activation of SHP-1 has been reported to play a role in diminishing immune responses through inhibition of inflammatory cytokines in response to *Leishmania* infections [118]. In knockout models of SHP-1, an increase in NF-κB transcription was observed suggesting that SHP-1 may be involved in regulating the translocation of NF-κB to the nucleus [119]. Increases in SHP-1 are also observed in diabetic retinas, and may be translatable to the cardiac system where overexpression of SHP-1 may impair NF-κB activity [120]. Although NF-κB activity was altered in the STZ rat models, it did not disrupt other deleterious cellular mechanisms including ROS generation and apoptosis.

Furthermore, our findings lead us to the concept that type II DM is closely linked to the NF-κB-dependent inflammatory processes. Type II diabetes evidently plays an important role in the phosphorylation of NF-κB. Phosphorylation of NF-κB at the serine 536 site has been associated with NF-κB activation leading to enhanced transactivation of the inflammatory marker [121]. As NF-κB activation is potentiated through phosphorylation, the total levels are also found to increase. NF-κB activation can enhance the transcription of inflammatory genes such as TNF-α and IL-6 [122–124]. Elevated levels of both phosphorylated and total forms of NF-κB clearly indicate the effect of type II DM on inflammation. Prolonged NF-κB activation has been linked to both ROS generation and cellular apoptosis that has been observed in this study on the *db*/*db* mouse model.

4.3. Exogenous oxytocin administration reverses the upregulation of RAGE expression

Administration of oxytocin in the condition of DM was capable of reducing RAGE protein expression. Expression of RAGE was significantly decreased to near control levels in the left ventricle of both type I and II diabetic animal models. Some evidence indicated that oxytocin can directly regulate RAGE concentration on the cells surface. It has been shown by Metz et al. [101], that activation of GPCRs in HEK 293 cells led to the cleavage of RAGE by a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and other matrix metalloproteinases (MMPs). The shedding phenomenon generates the extracellular, soluble subset of RAGE known as sRAGE that can act as a decoy molecule and bind to excess AGEs. The AGE interaction with sRAGE disrupts downstream signalling and prevents the initiation of nuclear transcription factors and the formation of ROS (Figure 17). On the basis of these observations and the data from this study, we postulate that RAGE shedding may be one of the primary mechanisms by which oxytocin is providing a protective effect against inflammatory processes in diabetes.

There are many contradictory studies on the use of sRAGE as a novel biomarker for DM or perhaps other chronic diseases. Studies by Selvin et al. [125] and Shemirani et al. [126], suggest that inflammation (measured through elevated C reactive protein (CRP) levels) and elevated reactive oxygen species, respectively, are directly linked to low plasma sRAGE. In order to further investigate the efficacy of oxytocin treatment, it would be of interest to study the plasma levels of sRAGE. Cleavage of RAGE through oxytocin action should lead to increased plasma concentrations of sRAGE but more reliable detection methods for the quantitation of sRAGE in *in vivo* models are required.

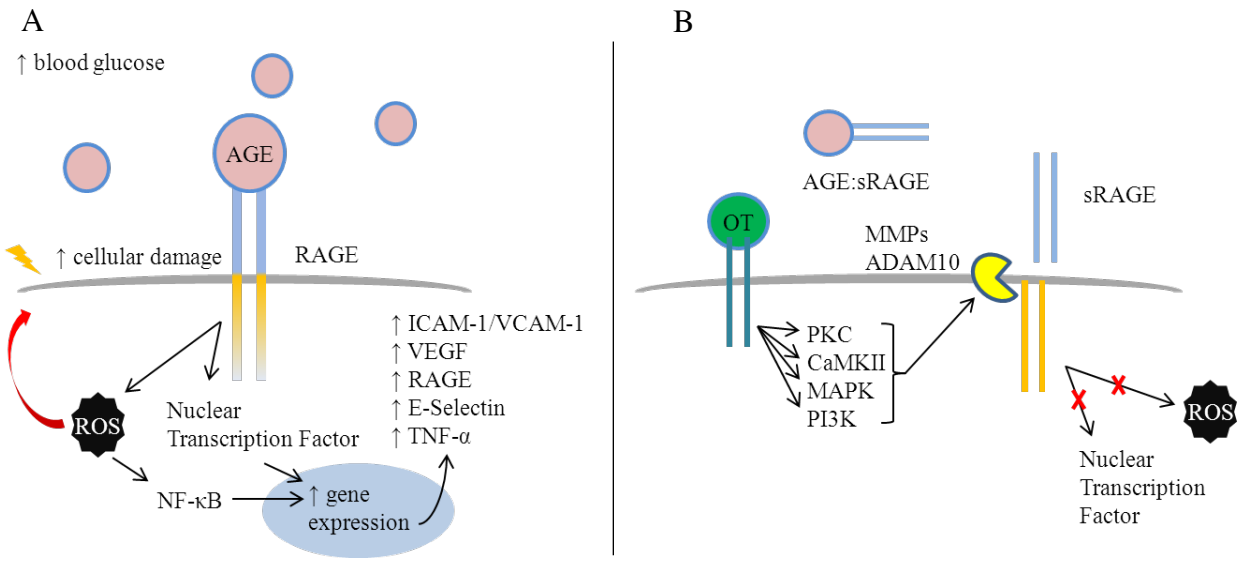


Figure 17: Mechanism of anti-inflammatory action by oxytocin. A) Elevated blood glucose levels are optimal for the formation of AGEs through protein glycation. Activation of RAGE triggers the up-regulation of nuclear transcription factors and ROS formation. These factors can increase expression of NF-κB leading to a positive feedback loop generating higher levels of RAGE, ICAM-1/VCAM-1, VEGF, E-selectin and TNFα. These factors are major players in the development of CVD. B) Administration of oxytocin and activation of the oxytocin receptor can trigger a series of downstream cascades including PKC, CaMKII, MAP kinases and PI3K. Signalling down these pathways can lead to activation of MMPs and ADAM10, which can proteolytically cleave the RAGE receptor. Ligation of sRAGE to AGEs can generate a conjugate incapable of activating the full-length receptor. Disruption of this signalling cascade with the use of oxytocin can provide the protection necessary against CVD.

4.4 Disruption of RAGE signalling cascade reduces reactive oxygen species accumulation and apoptosis in models of type II diabetes but not in type I diabetes

Reactive oxygen species generation is highly regulated by RAGE activation and signalling. DM has been associated with an imbalance of oxidants and anti-oxidants in the body. Signal transduction through the RAGE pathway leads to increases in ROS generation which can further disrupt the oxidant/anti-oxidant equilibrium [69]. RAGE dependent activation of NF-κB can further augment the formation of ROS leading to the observed increases in accumulation seen in the left ventricle of type I and II diabetic animals. Although oxytocin is capable of reducing RAGE expression in type I diabetes, the actions on ROS generation do not appear to be affected. Similarly, apoptosis was not reduced with oxytocin treatment, which is indicative of persistent damage. Type II diabetic animals treated with oxytocin recovered from the accumulation of ROS as well as from cell apoptosis in the left ventricle.

Since type I diabetes is considered to be of a primarily autoimmune origin, the mechanism of inflammation differs from those of type II diabetes. The complementary effects of autoimmune T-cell activation and inflammation leads to ß cell death [127]. Some studies have demonstrated the functionality of oxytocin receptors in T-lymphocytes [128]. Treatment with oxytocin may play a role in diminishing the inflammatory processes, but there are no studies on its actions on T-cell activity in diabetes. Cytotoxic T-cell mediated activity found in type I DM can act through the perforin/granzyme system which has been shown to elevate ROS formation in the mitochondria [127, 129]. Activation of the perforin/granzyme system is consistent with increased signalling through the apoptotic pathway as observed in the type I diabetic animals [129]. It suggests that oxytocin administration in type I diabetic animals may only provide partial protection through its anti-inflammatory effects but not through modulation of T-cell activity.

In the case of type II DM, a predominantly inflammatory disorder, the downregulation of ROS through oxytocin can be associated with reductions in RAGE expression and a blockade of NF-κB. As expected, the reduction in NF-κB expression can dampen the pro-oxidative state of cardiomyocytes. The reversal from the pro-apoptotic state in conditions of type II diabetes further supports this and highlights the importance of balancing oxidants and anti-oxidants. Oxytocin appears to provide protection against both ROS production and apoptotic signals in conditions of type II DM.

4.5 Protective mechanisms activated by oxytocin

Cardioprotection through administration of oxytocin has been well established. Previous work performed by our lab has elucidated some of the potential protective pathways through activation of Akt, ERK 1/2 and eNOS [81, 91, 100]. Incubation of H9c2 cells with glyceraldehyde-AGEs for short periods of time (up to 24 hours) was not detrimental to cell viability making it a suitable model to study AGE induced signalling mechanisms in the heart. Findings in H9c2 cells were similar to observations in our type II diabetic models suggesting that *in vitro* AGE stimulation can be used to simulate diabetic conditions. NF-κB phosphorylation was amplified demonstrating that it is activated shortly after exposure to AGEs initiating the inflammatory process. NF-κB activation occurs in two phases, the acute response within the first 48 hours, independent of mRNA and protein synthesis, followed by the chronic response at times after 48 hours, which is dependent on *de novo* synthesis of NF-κB [130]. Persistent activation of NF-κB leads to the deterioration of cardiac health and intensifies the inflammatory response.

The use of oxytocin as an anti-inflammatory agent has been established, but the mechanism behind its actions remain unclear [99]. In myocardial infarctions, elevations of pro-

inflammatory cytokines (TNF-α, IL-1β and IL-6) were reduced by oxytocin infusion [99]. The interaction between RAGE and its ligands may be a direct pathway triggering the inflammatory response accelerating tissue damage through the sustained release and activation of TNF-α, IL-1, IL-6 and metalloproteinases [131]. A blockade of the RAGE signalling pathway through the exogenous administration of sRAGE has been shown to be beneficial in reducing the inflammatory processes through decreases particularly in TNF-α and other metalloproteinases [131, 132]. Administration of sRAGE has further been shown to resolve dysfunctional NO synthesis.

The AGE-RAGE axis has been reported to be key component in modulating many cardiac pathologies, including myocardial infarctions [133]. NO, a component essential for cardiac function is produced by three isoforms of nitric oxide synthase (NOS), eNOS, neuronal NOS (nNOS) and inducible NOS (iNOS). Recruitment of pro-inflammatory cytokines can enhance iNOS expression [134]. Chronic overexpression of iNOS has been implicated in cardiac dysfunction, necrotic cell death, electrical abnormalities and protein tyrosine nitration [135, 136]. sRAGE administration to block the RAGE pathway is capable of preventing increases in iNOS expression and decreasing oxidative injury [133].

Our study shows that the use of oxytocin following AGE stimulus has a propensity towards a diminished acute response that is initiated by an AGE dependent activation of RAGE. Oxytocin can act as a blockade against the signalling cascade that perpetuates the development of chronic inflammation. Vascular, renal and cardiovascular damage can occur during extensive exposure to AGEs such as in the cases of DM. Early termination of the acute NF-κB response may be capable in preventing synthesis of mRNA and maturation of protein. Further

experimentation with increased sample sizes are needed to draw definitive conclusions on the mechanistic behavior of oxytocin action on RAGE and NF-κB.

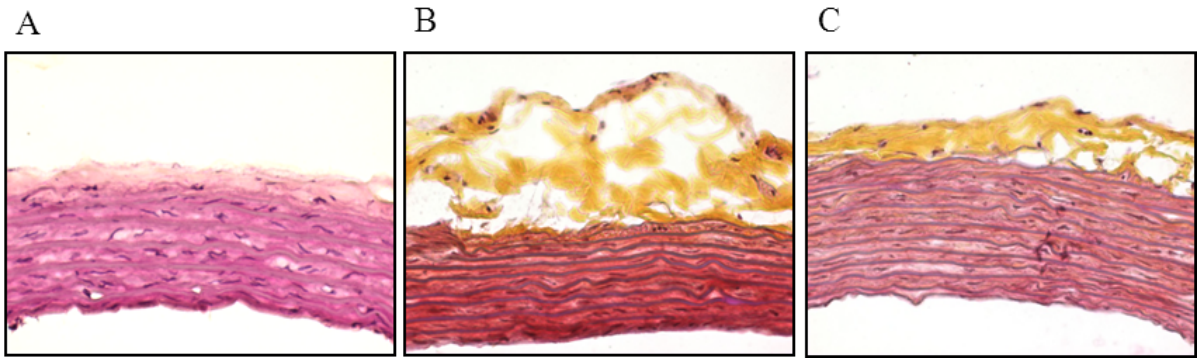
4.6 Current therapeutics for inflammation

Currently, there is a challenge to produce novel therapeutics for the treatment of inflammatory disorders. With the numerous players involved in inflammation, traditional treatments often work on specific pathways. Classical treatments against inflammation include the use of anti-inflammatory drugs including corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs). These drugs largely target the prostaglandin system through inhibition of upstream factors or protein synthesis [50, 137, 138]. With such a complex system, it is difficult to regulate the inflammatory pathway as there is a fine balance between the immune response against invading pathogens and preventing the development of disease caused by chronic systemic inflammation. With diabetes and cardiovascular disease closely related, targeting a common pathway may be the most effective approach. The use of oxytocin may be beneficial in these conditions as a multi-targeted approach for therapy. Harnessing the anti-inflammatory and anti-hyperglycemic effects of oxytocin may be invaluable for future therapeutics. Further studies on the mechanism of actions would be of interest to confirm its potential for use as monotherapy or in conjunction with other therapeutics for DM.

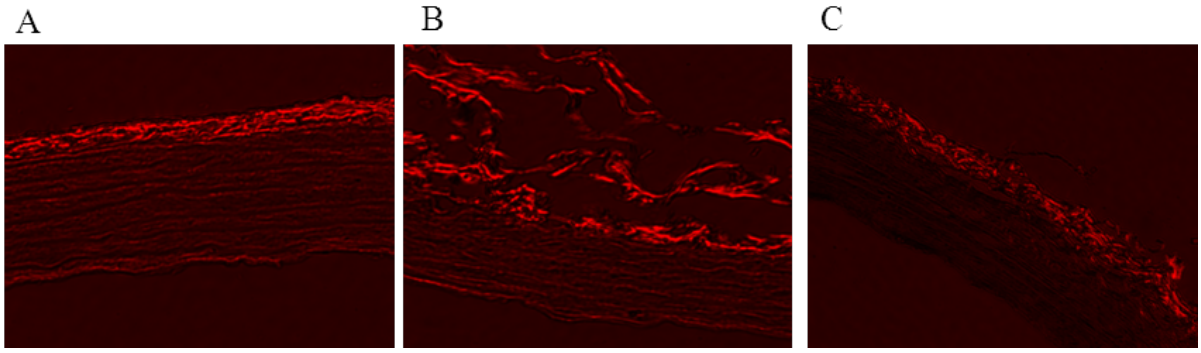
5. Conclusions

The results from this study support the direct and indirect cardioprotective effects of oxytocin. Direct action of oxytocin is capable of protecting from the metabolic changes in glucose regulation when subjected to diabetic conditions. Oxytocin increases glucose uptake in cells and encourages faster recovery to baseline blood glucose levels. Indirect action of oxytocin induced cardioprotection stems from its role as a signal blockade in RAGE and NF-κB. Obstruction of RAGE is found to be effective in both type I and II diabetes mellitus. Downstream inflammatory signal blockade by oxytocin infusion is more effective in the inflammation implicated type II diabetes. The pluripotent effects of oxytocin through its actions in reversing deleterious modifications to the heart and disrupting the inflammatory signalling cascade suggests it holds strong therapeutic potential.

6. Supplementary Material



Supplementary Figure 1: Periodic-acid Schiff stain of rat aorta for polysaccharides. A) Control B) Streptozotocin, vehicle treated C) Streptozotocin with oxytocin treatment.



Supplementary Figure 2: Picrosirius red staining of rat aorta for collagen viewed under polarized light. A) Control B) Streptozotocin, vehicle treated C) Streptozotocin with oxytocin treatment

7. References

1. Maritim A, Sanders R, Watkins J (2003) Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol 17:24–38. doi: 10.1002/jbt.10058

2. Zimmet P, Alberti K, Shaw J (2001) Global and societal implications of the diabetes epidemic. Nature 414:

3. Zeyda M, Stulnig TM (2009) Obesity, inflammation, and insulin resistance--a mini-review. Gerontology 55:379–86. doi: 10.1159/000212758

4. Hotamisligil GS (2006) Inflammation and metabolic disorders. Nature 444:860–7. doi: 10.1038/nature05485

5. Boyle JP, Thompson TJ, Gregg EW, et al. (2010) Projection of the year 2050 burden of diabetes in the US adult population: dynamic modeling of incidence, mortality, and prediabetes prevalence. Popul Health Metr 8:29. doi: 10.1186/1478-7954-8-29

6. US Department of Health and Human Services (2014) National Diabetes Statistics Report.

7. Pelletier C, Dai S, Roberts KC, et al. (2012) Report summary. Diabetes in Canada: facts and figures from a public health perspective. Chronic Dis Inj Can 33:53–4.

8. Himsworth H (1936) Diabetes mellitus: its differentiation into insulin-sensitive and insulin-insensitive types. Lancet 227:127–130.

9. Alberti KG, Zimmet PZ (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15:539–53. doi: 10.1002/(SICI)1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S

10. American Diabetes Association (2008) Diagnosis and classification of diabetes mellitus. Diabetes Care 31 Suppl 1:S55–60. doi: 10.2337/dc08-S055

11. McGibbon A, Richardson C, Hernandez C, Dornan J (2013) Pharmacotherapy in Type 1 Diabetes. Can J Diabetes 37:S56–S60. doi: 10.1016/j.jcjd.2013.01.020

12. Baekkeskov S, Aanstoot HJ, Christgau S, et al. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347:151–6. doi: 10.1038/347151a0

13. Nakayama M, Abiru N, Moriyama H, Babaya N (2005) Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 435:165–71. doi: 10.1038/nature03512

14. Chatenoud L (2010) Immune therapy for type 1 diabetes mellitus-what is unique about anti-CD3 antibodies? Nat Rev Endocrinol 6:149–57. doi: 10.1038/nrendo.2009.275

15. Wherrett DK, Bundy B, Becker DJ, et al. (2011) Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. Lancet 378:319–27. doi: 10.1016/S0140-6736(11)60895-7

16. Yale J (2008) Pharmacologic Management of type 2 diabetes. Can. J. Diabetes

17. Harper W, Clement M, Goldenberg R, et al. (2013) Pharmacologic Management of Type 2 Diabetes. Can J Diabetes 37:S61–S68. doi: 10.1016/j.jcjd.2013.01.021

18. Roep BO, Atkinson M (2004) Animal models have little to teach us about type 1 diabetes: 1. In support of this proposal. Diabetologia 47:1650–6. doi: 10.1007/s00125-004-1517-1

19. Roep BO, Atkinson M, von Herrath M (2004) Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. Nat Rev Immunol 4:989–97. doi: 10.1038/nri1502

20. Chen D, Wang M-W (2005) Development and application of rodent models for type 2 diabetes. Diabetes Obes Metab 7:307–17. doi: 10.1111/j.1463-1326.2004.00392.x

21. Rees DA, Alcolado JC (2005) Animal models of diabetes mellitus. Diabet Med 22:359–70. doi: 10.1111/j.1464-5491.2005.01499.x

22. Chatzigeorgiou A, Halapas A, Kalafatakis K, Kamper E (2009) The use of animal models in the study of diabetes mellitus. In Vivo 23:245–58.

23. Neubauer N, Kulkarni RN (2006) Molecular approaches to study control of glucose homeostasis. ILAR J 47:199–211.

24. Omar BA, Vikman J, Winzell MS, et al. (2013) Enhanced beta cell function and anti-inflammatory effect after chronic treatment with the dipeptidyl peptidase-4 inhibitor vildagliptin in an advanced-aged diet-induced obesity mouse model. Diabetologia 56:1752–60. doi: 10.1007/s00125-013-2927-8

25. Bugger H, Abel ED (2009) Rodent models of diabetic cardiomyopathy. Dis Model Mech 2:454–66. doi: 10.1242/dmm.001941

26. Yusuf S, Reddy S, Ounpuu S, Anand S (2001) Global Burden of Cardiovascular Diseases: Part I: General Considerations, the Epidemiologic Transition, Risk Factors, and Impact of Urbanization. Circulation 104:2746–2753. doi: 10.1161/hc4601.099487

27. Kannel W, McGee D (1979) Diabetes and cardiovascular disease. JAMA J. Am. Med. …

28. Mangiapane H (2012) Cardiovascular disease and diabetes. Adv Exp Med Biol 771:219–28. doi: 10.1007/978-1-4614-5441-0_17

29. Aronson D, Rayfield EJ (2002) How hyperglycemia promotes atherosclerosis: molecular mechanisms. Cardiovasc Diabetol 1:1.

30. Ross R (1999) Atherosclerosis--an inflammatory disease. N Engl J Med 340:115–26. doi: 10.1056/NEJM199901143400207

31. Tsai EC, Hirsch IB, Brunzell JD, Chait a (1994) Reduced plasma peroxyl radical trapping capacity and increased susceptibility of LDL to oxidation in poorly controlled IDDM. Diabetes 43:1010–4.

32. Li H, Li H, Bao Y, et al. (2011) Free fatty acids induce endothelial dysfunction and activate protein kinase C and nuclear factor- ϰ B pathway in rat aorta. Int J Cardiol 152:218–224. doi: 10.1016/j.ijcard.2010.07.019

33. Singh R, Barden a, Mori T, Beilin L (2001) Advanced glycation end-products: a review. Diabetologia 44:129–46. doi: 10.1007/s001250051591

34. Ahmed N (2005) Advanced glycation endproducts--role in pathology of diabetic complications. Diabetes Res Clin Pract 67:3–21. doi: 10.1016/j.diabres.2004.09.004

35. Vlassara H, Palace M (2002) Diabetes and advanced glycation endproducts. J. Intern. Med.

36. Basta G, Schmidt AM, De Caterina R (2004) Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc Res 63:582–92. doi: 10.1016/j.cardiores.2004.05.001

37. Busch M, Franke S, Ruster C, Wolf G (2010) Advanced glycation end products and the kidney. Eur J Clin Investig 40:742–755.

38. Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414:813–820.

39. Sakurai S, Yonekura H, Yamamoto Y, et al. (2003) The AGE-RAGE system and diabetic nephropathy. J Am Soc Nephrol 14:S259–63.

40. Bierhaus A, Humpert PM, Morcos M, et al. (2005) Understanding RAGE, the receptor for advanced glycation end products. J Mol Med (Berl) 83:876–86. doi: 10.1007/s00109-005-0688-7

41. Basta G (2008) Receptor for advanced glycation endproducts and atherosclerosis: From basic mechanisms to clinical implications. Atherosclerosis 196:9–21. doi: 10.1016/j.atherosclerosis.2007.07.025

42. Schmidt AM, Vianna M, Gerlach M, et al. (1992) Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. J Biol Chem 267:14987–97.

43. Malherbe P, Richards JG, Gaillard H, et al. (1999) cDNA cloning of a novel secreted isoform of the human receptor for advanced glycation end products and characterization of cells co-expressing cell-surface scavenger receptors and Swedish mutant amyloid precursor protein. Brain Res Mol Brain Res 71:159–70.

44. Hudson BI, Carter AM, Harja E, et al. (2008) Identification, classification, and expression of RAGE gene splice variants. FASEB J 22:1572–80. doi: 10.1096/fj.07-9909com

45. Ding Q, Keller JN (2005) Evaluation of rage isoforms , ligands , and signaling in the brain. 1746:18–27. doi: 10.1016/j.bbamcr.2005.08.006

46. Sakaguchi M, Murata H, Yamamoto K, et al. (2011) TIRAP, an adaptor protein for TLR2/4, transduces a signal from RAGE phosphorylated upon ligand binding. PLoS One 6:e23132. doi: 10.1371/journal.pone.0023132

47. Ramasamy R, Yan SF, Schmidt AM (2011) Receptor for AGE (RAGE): signaling mechanisms in the pathogenesis of diabetes and its complications. Ann N Y Acad Sci 1243:88–102. doi: 10.1111/j.1749-6632.2011.06320.x

48. Hudson BI, Kalea AZ, Del Mar Arriero M, et al. (2008) Interaction of the RAGE cytoplasmic domain with diaphanous-1 is required for ligand-stimulated cellular migration through activation of Rac1 and Cdc42. J Biol Chem 283:34457–68. doi: 10.1074/jbc.M801465200

49. Ryan G, Majno G (1977) Acute Inflammation. A review. Am. J. Pathol.

50. Vane J, Botting R (1987) Inflammation and the mechanism of action of anti-inflammatory drugs. FASEB J.

51. Libby P (2007) Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutr Rev. doi: 10.1301/nr.2007.dec.S140

52. Rabinovitch A, Suarez-Pinzon WL (2003) Role of cytokines in the pathogenesis of autoimmune diabetes mellitus. Rev Endocr Metab Disord 4:291–9.

53. Cnop M, Welsh N, Jonas J-C, et al. (2005) Mechanisms of Pancreatic -Cell Death in Type 1 and Type 2 Diabetes: Many Differences, Few Similarities. Diabetes 54:S97–S107. doi: 10.2337/diabetes.54.suppl_2.S97

54. Donath MY, Shoelson SE (2011) Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 11:98–107. doi: 10.1038/nri2925

55. Wellen K, Hotamisligil G (2005) Inflammation, stress, and diabetes. J Clin Invest 115:1111–1119. doi: 10.1172/JCI200525102.The

56. Eizirik DL, Colli ML, Ortis F (2009) The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol 5:219–26. doi: 10.1038/nrendo.2009.21

57. Hotamisligil G, Shargill N, Spiegelman B (1993) Adipose Expression of Tumor Necrosis Factor α : Direct Role in Obesity-Linked Insulin Resistance. Science (80-) 259:87–91.

58. Kern PA, Ranganathan S, Li C, et al. (2001) Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol Endocrinol Metab 280:E745–51.

59. Bierhaus A, Illmer T, Kasper M (1997) Advanced Glycation End Product (AGE)–mediated induction of tissue factor in cultured endothelial cells Is dependent on RAGE. Circulation

60. Barnes PJ, Karin M (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066–71. doi: 10.1056/NEJM199704103361506

61. Li J, Schmidt AM (1997) Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. J Biol Chem 272:16498–506.

62. Bast A, Haenen G, Doelman C (1991) Oxidants and antioxidants: state of the art. Am. J. Med. 91:

63. Cross CE, Halliwell B, Borish ET, et al. (1987) Oxygen radicals and human disease. Ann Intern Med 107:526–45.

64. Emerit J, Edeas M, Bricaire F (2004) Neurodegenerative diseases and oxidative stress. Biomed Pharmacother 58:39–46. doi: 10.1016/j.biopha.2003.11.004

65. Dhalla NS, Temsah RM, Netticadan T (2000) Role of oxidative stress in cardiovascular diseases. J Hypertens 18:655–73.

66. Baynes J (1991) Role of Oxidative Stress in Development of Complications in Diabetes. Diabetes 40:405–412.

67. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB (2010) Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med 49:1603–16. doi: 10.1016/j.freeradbiomed.2010.09.006

68. Hussain SP, Hofseth LJ, Harris CC (2003) Radical causes of cancer. Nat Rev Cancer 3:276–85. doi: 10.1038/nrc1046

69. Giardino I, Edelstein D, Brownlee M (1996) BCL-2 expression or antioxidants prevent hyperglycemia-induced formation of intracellular advanced glycation endproducts in bovine endothelial cells. J Clin Invest 97:1422–8. doi: 10.1172/JCI118563

70. Yu T, Sheu S-S, Robotham JL, Yoon Y (2008) Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. Cardiovasc Res 79:341–51. doi: 10.1093/cvr/cvn104

71. Nishikawa T, Edelstein D, Du XL, et al. (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787–90. doi: 10.1038/35008121

72. Craven P a, Phillips SL, Melhem MF, et al. (2001) Overexpression of manganese superoxide dismutase suppresses increases in collagen accumulation induced by culture of mesangial cells in high-media glucose. Metabolism 50:1043–8. doi: 10.1053/meta.2001.25802

73. Unoki H, Bujo H, Yamagishi S, et al. (2007) Advanced glycation end products attenuate cellular insulin sensitivity by increasing the generation of intracellular reactive oxygen species in adipocytes. Diabetes Res Clin Pract 76:236–44. doi: 10.1016/j.diabres.2006.09.016

74. Gimpl G, Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81:629–83.

75. Richard P, Moos F, Freund-Mercier M (1991) Central Effects of Oxytocin. Physiol. Rev.

76. Dale H (1906) On some physiological actions of ergot. J. Physiol.

77. Ott I, Scott JC (1910) The action of infundibulin upon the mammary secretion. Exp Biol Med 8:48–49. doi: 10.3181/00379727-8-27

78. Vrachnis N, Malamas FM, Sifakis S, et al. (2011) The oxytocin-oxytocin receptor system and its antagonists as tocolytic agents. Int J Endocrinol 2011:350546. doi: 10.1155/2011/350546

79. Zingg HH (1996) Vasopressin and oxytocin receptors. Baillieres Clin Endocrinol Metab 10:75–96. doi: 10.1016/S0950-351X(96)80314-4

80. Mukaddam-Daher S, Yin Y-L, Roy J, et al. (2001) Negative Inotropic and Chronotropic Effects of Oxytocin. Hypertension 38:292–296. doi: 10.1161/01.HYP.38.2.292

81. Gutkowska J, Jankowski M (2008) Oxytocin revisited: It is also a cardiovascular hormone. J Am Soc Hypertens 2:318–25. doi: 10.1016/j.jash.2008.04.004

82. Gutkowska J, Jankowski M, Lambert C, et al. (1997) Oxytocin releases atrial natriuretic peptide by combining with oxytocin receptors in the heart. Proc Natl Acad Sci U S A 94:11704–9.

83. Devost D, Wrzal P, Zingg HH (2008) Oxytocin receptor signalling. Prog Brain Res 170:167–76. doi: 10.1016/S0079-6123(08)00415-9

84. Danalache BA, Paquin J, Donghao W, et al. (2007) Nitric oxide signaling in oxytocin-mediated cardiomyogenesis. Stem Cells 25:679–88. doi: 10.1634/stemcells.2005-0610

85. Oakley RH, Laporte S a, Holt J a, et al. (2001) Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis*. J Biol Chem 276:19452–60. doi: 10.1074/jbc.M101450200

86. Zingg HH, Laporte S a. (2003) The oxytocin receptor. Trends Endocrinol Metab 14:222–227. doi: 10.1016/S1043-2760(03)00080-8

87. Holmes CL, Landry DW, Granton JT (2003) Science review: Vasopressin and the cardiovascular system part 1--receptor physiology. Crit Care 7:427–34. doi: 10.1186/cc2337

88. Jankowski M, Hajjar F, Kawas SA, et al. (1998) Rat heart: a site of oxytocin production and action. Proc Natl Acad Sci U S A 95:14558–63.

89. Gutkowska J, Jankowski M (2009) Oxytocin: Old Hormone, New Drug. Pharmaceuticals 2:168–183. doi: 10.3390/ph203168

90. Gutkowska J, Jankowski M (2012) Oxytocin revisited: its role in cardiovascular regulation. J Neuroendocrinol 24:599–608. doi: 10.1111/j.1365-2826.2011.02235.x

91. Işeri SO, Sener G, Sağlam B, et al. (2005) Oxytocin ameliorates oxidative colonic inflammation by a neutrophil-dependent mechanism. Peptides 26:483–91. doi: 10.1016/j.peptides.2004.10.005

92. Altszuler N, Hampshire J (1981) Oxytocin infusion increases plasma insulin and glucagon levels and glucose production and uptake in the normal dog. Diabetes 30:112–4.

93. Florian M, Jankowski M, Gutkowska J (2010) Oxytocin increases glucose uptake in neonatal rat cardiomyocytes. Endocrinology 151:482–91. doi: 10.1210/en.2009-0624

94. Bobbioni-Harsch E, Frütiger S, Hughes G (1995) Physiological con- centrations of oxytocin powerfully stimulate insulin secretion in vitro. Endocrine 1:55–59.

95. Zhang H, Wu C, Chen Q, et al. (2013) Treatment of obesity and diabetes using oxytocin or analogs in patients and mouse models. PLoS One 8:e61477. doi: 10.1371/journal.pone.0061477

96. Takeuchi M, Makita Z, Bucala R, et al. (2000) Immunological evidence that non-carboxymethyllysine advanced glycation end-products are produced from short chain sugars and dicarbonyl compounds in vivo. Mol Med 6:114–25.

97. Yu L, Zhao Y, Xu S, et al. (2013) Advanced Glycation End Product (AGE)-AGE Receptor (RAGE) System Upregulated Connexin43 Expression in Rat Cardiomyocytes via PKC and Erk MAPK Pathways. Int J Mol Sci 14:2242–57. doi: 10.3390/ijms14022242

98. Lee ES, Uhm K-O, Lee YM, et al. (2008) Oxytocin stimulates glucose uptake in skeletal muscle cells through the calcium-CaMKK-AMPK pathway. Regul Pept 151:71–4. doi: 10.1016/j.regpep.2008.05.001

99. Jankowski M, Bissonauth V, Gao L, et al. (2010) Anti-inflammatory effect of oxytocin in rat myocardial infarction. Basic Res Cardiol 105:205–218. doi: 10.1007/s00395-009-0076-5

100. Gonzalez-Reyes A (2012) Protective Signaling of Oxytocin in an in vitro Model of Myocardial Ischemia-Reperfusion. University of Montreal

101. Metz V V, Kojro E, Rat D, Postina R (2012) Induction of RAGE Shedding by Activation of G Protein-Coupled Receptors. PLoS One 7:e41823. doi: 10.1371/journal.pone.0041823

102. Noiseux N, Borie M, Desnoyers A, et al. (2012) Preconditioning of stem cells by oxytocin to improve their therapeutic potential. Endocrinology 153:5361–72. doi: 10.1210/en.2012-1402

103. Eiselein L, Schwartz HJ, Rutledge JC (2004) The challenge of type 1 diabetes mellitus. ILAR J 45:231–6.

104. Björkstrand E, Uvnäs-Moberg K (1996) Central oxytocin increases food intake and daily weight gain in rats. Physiol Behav 59:947–52.

105. Krause MP, Riddell MC, Hawke TJ (2011) Effects of type 1 diabetes mellitus on skeletal muscle: clinical observations and physiological mechanisms. Pediatr Diabetes 12:345–64. doi: 10.1111/j.1399-5448.2010.00699.x

106. Wohaieb S a, Godin D V (1987) Alterations in free radical tissue-defense mechanisms in streptozocin-induced diabetes in rat. Effects of insulin treatment. Diabetes 36:1014–8.

107. Zhang G, Cai D (2011) Circadian intervention of obesity development via resting-stage feeding manipulation or oxytocin treatment. Am J Physiol … 1004–1012. doi: 10.1152/ajpendo.00196.2011.

108. Steinberg G, Kemp B (2009) AMPK in health and disease. Physiol Rev 1025–1078. doi: 10.1152/physrev.00011.2008.

109. Bilim O, Takeishi Y, Kitahara T, et al. (2008) Diacylglycerol kinase zeta inhibits myocardial atrophy and restores cardiac dysfunction in streptozotocin-induced diabetes mellitus. Cardiovasc Diabetol 7:2. doi: 10.1186/1475-2840-7-2

110. An D, Rodrigues B (2006) Role of changes in cardiac metabolism in development of diabetic cardiomyopathy. Am J Physiol Heart Circ Physiol 291:H1489–506. doi: 10.1152/ajpheart.00278.2006

111. Gutkowska J, Broderick TL, Bogdan D, et al. (2009) Downregulation of oxytocin and natriuretic peptides in diabetes: possible implications in cardiomyopathy. J Physiol 587:4725–36. doi: 10.1113/jphysiol.2009.176461

112. Hayat S a, Patel B, Khattar RS, Malik R a (2004) Diabetic cardiomyopathy: mechanisms, diagnosis and treatment. Clin Sci (Lond) 107:539–57. doi: 10.1042/CS20040057

113. Purcell NH, Tang G, Yu C, et al. (2001) Activation of NF-kappa B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. Proc Natl Acad Sci U S A 98:6668–73. doi: 10.1073/pnas.111155798

114. Nation DA, Szeto A, Mendez AJ, et al. (2010) Oxytocin attenuates atherosclerosis and adipose tissue inflammation in socially isolated ApoE-/- mice. Psychosom Med 72:376–82. doi: 10.1097/PSY.0b013e3181d74c48

115. Parathath S, Grauer L, Huang L-S, et al. (2011) Diabetes adversely affects macrophages during atherosclerotic plaque regression in mice. Diabetes 60:1759–69. doi: 10.2337/db10-0778

116. Mollah ZU a., Pai S, Moore C, et al. (2008) Abnormal NF-kB Function Characterizes Human Type 1 Diabetes Dendritic Cells and Monocytes. J Immunol 180:3166–3175. doi: 10.4049/jimmunol.180.5.3166

117. Neznanov N, Neznanova L, Kondratov R V, et al. (2004) The ability of protein tyrosine phosphatase SHP-1 to suppress NFkappaB can be inhibited by dominant negative mutant of SIRPalpha. DNA Cell Biol 23:175–82. doi: 10.1089/104454904322964779

118. Forget G, Matte C, Siminovitch K a, et al. (2005) Regulation of the Leishmania-induced innate inflammatory response by the protein tyrosine phosphatase SHP-1. Eur J Immunol 35:1906–17. doi: 10.1002/eji.200526037

119. Massa PT, Wu C (1998) Increased inducible activation of NF-kappaB and responsive genes in astrocytes deficient in the protein tyrosine phosphatase SHP-1. J Interferon Cytokine Res 18:499–507.

120. Giacco F, Brownlee M (2010) Oxidative stress and diabetic complications. Circ Res 107:1058–1070. doi: 10.1161/CIRCRESAHA.110.223545.Oxidative

121. Viatour P, Merville M-P, Bours V, Chariot A (2005) Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci 30:43–52. doi: 10.1016/j.tibs.2004.11.009

122. Morita M, Yano S, Yamaguchi T, Sugimoto T (2013) Advanced glycation end products-induced reactive oxygen species generation is partly through NF-kappa B activation in human aortic endothelial cells. J Diabetes Complications 27:11–5. doi: 10.1016/j.jdiacomp.2012.07.006

123. Yan S Du, Yan S, Chen X, Fu J (1995) Non-enzymatically glycated tau in Alzheimer's disease induces neuronal oxidant stress resulting in cytokine gene expression and release of amyloid β-peptide. Nat. Med.

124. Hoffmann A, Baltimore D (2006) Circuitry of nuclear factor κB signaling. Immunol. Rev.

125. Selvin E, Halushka M, Rawlings A (2013) sRAGE and risk of diabetes, cardiovascular disease and death. Diabetes 1–27.

126. Shemirani F, Yazdanparast R (2014) The interplay between hyperglycemia-induced oxidative stress markers and the level of soluble receptor for advanced glycation end products (sRAGE) in K562 cells. Mol Cell Endocrinol. doi: 10.1016/j.mce.2014.05.019

127. Donath MY, Størling J, Maedler K, Mandrup-Poulsen T (2003) Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. J Mol Med (Berl) 81:455–70. doi: 10.1007/s00109-003-0450-y

128. Ndiaye K, Poole DH, Pate JL (2008) Expression and regulation of functional oxytocin receptors in bovine T lymphocytes. Biol Reprod 78:786–93. doi: 10.1095/biolreprod.107.065938

129. Pardo J, Bosque A, Brehm R, et al. (2004) Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. J Cell Biol 167:457–68. doi: 10.1083/jcb.200406115

130. Bierhaus A, Schiekofer S, Schwaninger M, et al. (2001) Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. Diabetes 50:2792–808.

131. Schmidt A, Yan S Du (2001) The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin … 108:949–955. doi: 10.1172/JCI200114002.The

132. Lalla E, Lamster IB, Feit M, et al. (2000) Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. J Clin Invest 105:1117–24. doi: 10.1172/JCI8942

133. Bucciarelli LG, Kaneko M, Ananthakrishnan R, et al. (2006) Receptor for advanced-glycation end products: key modulator of myocardial ischemic injury. Circulation 113:1226–34. doi: 10.1161/CIRCULATIONAHA.105.575993

134. Csont T, Viappiani S, Sawicka J, et al. (2005) The involvement of superoxide and iNOS-derived NO in cardiac dysfunction induced by pro-inflammatory cytokines. J Mol Cell Cardiol 39:833–40. doi: 10.1016/j.yjmcc.2005.07.010

135. Yang X, Chowdhury N, Cai B, et al. (1994) Induction of myocardial nitric oxide synthase by cardiac allograft rejection. J Clin Invest 94:714–21. doi: 10.1172/JCI117390

136. Szabolcs M, Michler RE, Yang X, et al. (1996) Apoptosis of cardiac myocytes during cardiac allograft rejection. Relation to induction of nitric oxide synthase. Circulation 94:1665–73. doi: 10.1161/01.CIR.94.7.1665

137. Hong SL, Levine L (1976) Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory corticosteroids. Proc Natl Acad Sci U S A 73:1730–4.

138. Patrono C, Baigent C (2014) Nonsteroidal anti-inflammatory drugs and the heart. Circulation 129:907–16. doi: 10.1161/CIRCULATIONAHA.113.004480