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Dear Paul

SUMMER RESEARCH SCHOLARSHIPS - 2014-2015

Further to previous correspondence we enclose the hard copy report of The Otago Diabetes Research Trust supported scholar for 2014 - 2015, Hazel M. Nissen.

On behalf of the Foundation we thank The Otago Diabetes Research Trust for their continuing contribution to Summer Research Scholarships.

Please contact the writer if you have any queries.

Yours sincerely
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How does diabetes affect the microcirculation of the heart?

Specialised Title: The contribution of coronary microvascular dysfunction to cardiac complications in type 2 diabetes mellitus

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Abstract

Diabetes Mellitus (DM) is strongly associated with cardiovascular disease and is escalating worldwide. DM-induced disease of small heart vessels, the coronary microvessels, contributes to increased cardiovascular morbidity and mortality in diabetic patients. However, our limited ability to directly measure coronary microcirculation restricts progress in our understanding of DM heart disease. I aimed to establish an innovative tool, vascular casting, to measure coronary microvascular perfusion and investigate how type 2 DM impairs this. My study supports the validity of this technique, demonstrates its potential to enhance our understanding of coronary microcirculation in DM, and generates novel opportunities for future cardiovascular research.

Introduction

Identifying and understanding cardiovascular complications associated with diabetes mellitus (DM) is critical in optimising treatments for this rapidly increasing patient population. The global prevalence of DM is rising; in 2013 it was estimated that 382 million people had the disease, and it is predicted to reach 592 million by 2035[1]. Diabetic patients have increased cardiovascular morbidity and mortality compared to non-diabetic patients; which not only increases suffering of the diabetic patient, but are also social and economic burdens[2]. DM is characterised by hyperglycaemia (fasting plasma glucose ≥7.0mM or glycated haemoglobin >6.5%), arising from defective insulin secretion, action, or both[3]. Type 2 DM is the prevalent form (85 to 95% of all diabetes types) and is characterised by a combination of insulin resistance and defective insulin secretion[2].

DM-induced disease of the small vessels of the heart, the coronary microvessels, has been shown to contribute to increased cardiovascular morbidity and mortality[4,5]. These microvessels have a critical role in regulating blood pressure and flow for efficient oxygen and nutrient delivery to meet the high metabolic demands of the working heart muscle[6]. Dysfunction of the microvessels involves increased rigidity and permeability as well as dysfunctional autoregulation of blood flow[7,8]. This microvascular dysfunction precedes development of overt coronary artery disease[4]. Consequently, understanding the structure and the function of the coronary microcirculation, and particularly during DM, is a key factor in understanding cardiovascular complications in type 2 diabetic patients.

Despite the critical role of microvascular perfusion in the diabetic heart, our limited ability to measure coronary microvascular perfusion directly has restricted the progress in our understanding of the pathology of DM heart disease. Therefore, having tools to measure microvascular perfusion directly is crucial. To address this, my Honours project in 2014 assessed the viability of an existing

microvascular perfusion technique for its specific use in the coronary microcirculation. Isolated rat hearts were exposed to different physiological stimuli, before the microvascular structure was rapidly preserved as a vascular cast, as has previously been described in skeletal muscle[9]. While representative casts appeared to reflect changes in microvascular perfusion, quantitative analysis remained to be established. Thus, in order for me to complete the establishment of this new innovative tool and gain valuable information on how DM impairs coronary microvascular perfusion, I aimed in my summer research project to:

1) Develop a method for analysing the casts and to test the feasibility of this technique in measuring changes in coronary microvascular perfusion.

2) Apply the developed techniques in a pilot study to examine coronary microvascular perfusion in type 2 DM hearts under basal and metabolic stress conditions.

My hypothesis was that analysis of the casts would detect changes in coronary microvascular perfusion, and that the diabetic heart has impaired regulation of microvascular perfusion.

Methods

Vascular casts were produced under a range of test conditions. Isolated hearts from Sprague-Dawley rats (n=9) were cast under basal conditions (controls), as well as under pharmacological-induced dilation by increased metabolic demand (isoproterenol $1x10^{-8}$M) or under vasoconstriction (angiotensin II $1x10^{-7}$M). Secondarily, non-diabetic and type 2 diabetic hearts from Zucker Diabetic Fatty (ZDF) rats were cast under basal conditions (n=1) and isoproterenol $1x10^{-8}$M (n=2). The type 2 diabetic ZDF rats have a homozygous mutation in the gene for the leptin receptor, leading to obesity, insulin resistance, and a predisposition to DM [10,11]. Coronary vascular casts were produced using Vertex Cold Curing Denture Repair Material (methyl methacrylate; Dentimex, Zeist, Holland), mixed to a low viscosity and rapidly infused into the arterial tree of isolated perfused beating rat hearts. This cured within minutes and preserved the arterial structure at one of the

physiological time points (see Figure 1 below). After digestion of the heart tissue with 1M KOH for 48 hours, the cast revealed a representation of the extent of microvascular perfusion.

The casts were imaged using a Skyscan 1172 micro-computerised tomography (μCT) X-ray scanner with a 10-megapixel digital camera (Bruker-MicroCT, Kontich, Belgium) at a resolution of 7μm per pixel and X-ray intensity of 30mV. This permitted 3-dimensional imaging, and investigation of the degree of branching and size of the coronary arteries. The image stacks were then analysed using ImageJ (1.46r and 1.49j10) software (National Institute of Health, Maryland, USA).

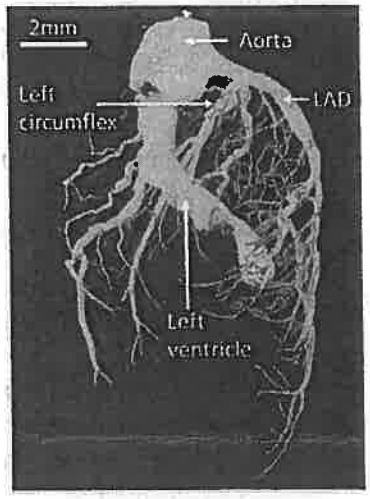

Figure 1: ***Micro-computerised tomography scan of a cast of the coronary arteries of an isolated rat heart with simple neurite tracing.*** *The arteries highlighted in pink branch off the left anterior descending (LAD) artery and depict those that were analysed with simple neurite tracing.*

In order to measure the degree of branching, the plugin Simple Neurite Tracer[12] was used in ImageJ (1.49j10). Due to the extensive branching of the casts, this project focussed on the left anterior descending (LAD) artery, as it is critical in supplying the left ventricle; the main pump of the heart. Thus, for analysis purposes the LAD was considered the primary (1°) artery, and all downstream branches except the left circumflex artery were documented (Figure 1). The arteries were traced through the image stack, and branches directly off the LAD were considered 2°, and those branching directly off the 2° arteries were considered 3°, and so on. The numbers of branches at each level were then counted manually.

Cast volumes incorporating the aorta and left ventricle were excluded from the analysis. The volume of the coronary circulation was then measured in ImageJ (1.46r) software. The *optimise threshold* function in the plugin BoneJ (version 1.3.14)[13] was used to autothreshold the cast image into black (background) and white (foreground) from a histogram of the image stack. The image was processed by the *volume fraction* function, which calculated the proportion of the image volume that was foreground and thus the volume of the cast.

Data were graphed in GraphPad Prism (version 6, GraphPad Software, Inc., CA, USA). Due to the small sample sizes no statistical analyses were performed.

Results

The amount of branching increased between 1°, 2°, 3° and 4° levels (Figure 2A), whereas at the 5° and 6° levels the amount of branching decreased.

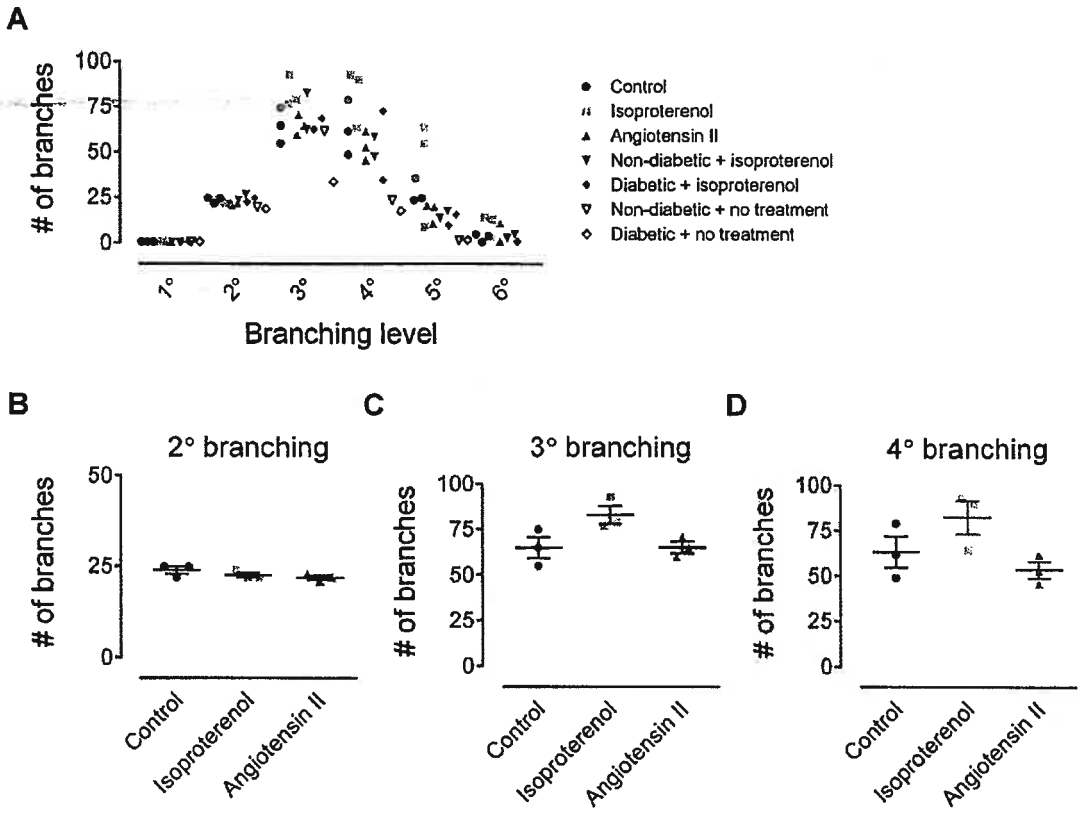

Figure 2: ***Number of branches at each level off the left anterior descending artery in casts of the coronary circulation of isolated rat hearts with different treatments.*** *Treatments include type 2 diabetic and non-diabetic ZDF rat hearts with (n=2) or without (n=1) $1x10^{-8}M$ isoproterenol, and Sprague Dawley rat hearts (n=3) with either no treatment (controls), $1x10^{-8}M$ isoproterenol, or $1x10^{-7}M$ angiotensin II. (A) Overall branch count at each level for all casts; (B) Number of 2° branches; (C) number of 3° branches; and (D) number of 4° branches in the Sprague-Dawley rats with pharmacological treatment. Individual data are presented with the mean ± SEM.*

The number of branches was very similar between groups at the 1° and 2° branching levels (the main conduction arteries) (Figure 2A+B). However, at the 3° and 4° levels (small resistance

arteries and arterioles) (Figure 2C+D), the degree of branching suggests differing degrees of microvascular perfusion. Although statistical analysis was unable to be performed with this small sample size, at the 3° level the mean number of branches increased with isoproterenol relative to controls. Furthermore, at the 4° level the mean number of branches increased with isoproterenol and decreased with angiotensin II treatment relative to controls.

Preliminary data from both diabetic and non-diabetic ZDF hearts is suggestive of recruitment of increased microvascular branches following treatment with isoproterenol (Figure 3). However, the small sample size limits conclusions about whether microvascular perfusion is impaired in type 2 DM under basal or stressful (isoproterenol) conditions.

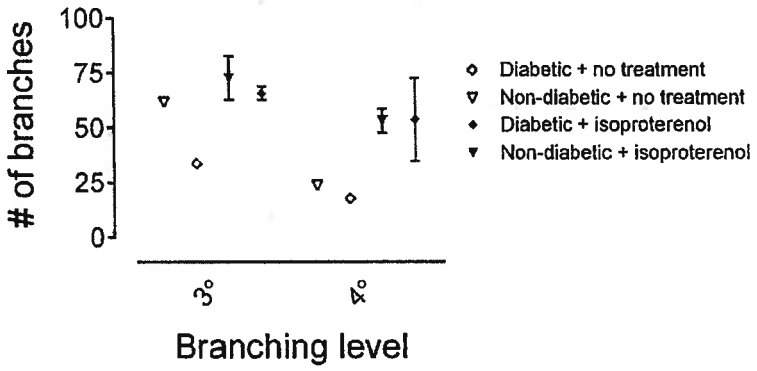

Figure 3: Number of 3° and 4° branches off the left anterior descending artery in casts of the coronary circulation of non-diabetic and diabetic isolated ZDF rat hearts. Non-diabetic and diabetic hearts with no treatment (n=1) or $1x10^{-8}$M isoproterenol treatment (n=2). Data are expressed individually or as the mean ± SEM.

Analysis of overall cast volume showed no differences between groups (Figure 4). Although conclusions are restricted by the limited sample sizes, this data doesn't support total volume assessment as a measure of microvascular perfusion.

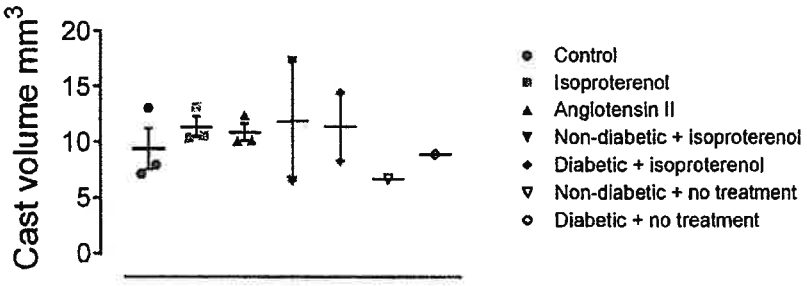

Figure 4: Volumes of casts of the coronary circulation from isolated rat hearts under different conditions. Control, $1x10^{-8}$M isoproterenol, and $1x10^{-7}$M angiotensin II (n=3); non-diabetic and type 2 diabetic ZDF with no treatment (n=1) or $1x10^{-8}$M isoproterenol (n=2). Individual values are presented with the mean ± SEM.

Discussion

This project aimed to examine methods for the analysis of vascular casts of isolated rat hearts, and to assess the validity of this technique for determining DM-induced changes in coronary microvascular perfusion. Using μCT and image analysis software I was able to determine numbers of vessels at each branching level. The extent of branching of the vascular casts reflected pharmacological-induced changes in microvascular perfusion. Although the sample size of the available data is not sufficient to draw any robust conclusions on how type 2 DM impairs coronary microvascular perfusion, the data thus far supports the validity of using this technique for detecting such changes. Therefore, this study demonstrated the potential of this technique as a valuable tool to measure coronary microvascular perfusion under different physiological or pathophysiological conditions.

The previous use of this vascular casting technique in the constant perfused rat hindlimb showed increased amounts of vessels filled with a low dose of norepinephrine without a change in cast weight, wheras the vasoconstrictor serotonin showed a decreased amount of filled vessels and cast weight[9]. I found differing counts of 3° and 4° branching following pharmacological hemodynamic manipulations suggesting this technique is able to detect changes in microvascular perfusion. However, whether this is due to changes in the diameter of the perfused vessels (and thus

permitting the casting compound to penetrate) or due to recruitment of new vessels previously not perfused is unclear and requires further study. The decrease in the amount of branching at the 5° and 6° levels suggests that at these levels the accuracy of the technique may be reduced, which might be improved by adjusting the constitution of the dental acrylic. Thus far, analysis of the total volumes of the coronary casts is inconclusive, but suggests it does not reflect changes in microvascular perfusion. It is important to note that current volume analysis incorporated both large conductance vessels as well as microvessels. Restricting examination of cast volume to specific coronary regions may provide a potential approach to improve utility of this measure.

The methods I developed to analyse the casts differ from those used by Newman, et al. [9] in the constant perfused rat hind limb from which the vascular casting techniques were adapted. Newman, et al. [9] determined the degree of vascular casting (vessel number and volume) by scanning photographic white on black images. The amount of white pixels was then normalised to the ratio of the cast mass to rat body mass. In this study, the μCT imaging combined with imaging software permitted detailed 3-dimensional analysis of the casts. In future, this technique may also benefit from normalising the data to the heart mass to remove any potential effects of heart size on the amount of branching.

The results of this study can also be correlated to available data on the haemodynamics and cardiac function from each of the hearts. This could reveal valuable information on how these parameters relate to coronary microvascular perfusion in the controlled isolated heart environment.

Vascular casting of isolated hearts combined with μCT imaging generates opportunities for prospective studies to further examine coronary microvascular perfusion. For example, by investigating the degree of LAD branching, future studies could compare epi- and endocardial microvascular perfusion. Furthermore, increased application of this technique may aid in investigation of other parameters such as the diameter and length of the vessels, or further pharmacological or pathological conditions (for example stenosis). Thus, this innovative vascular

casting tool adds to the limited repertoire of techniques available to measure microvascular perfusion in a beating heart, and it would be worthwhile to optimise this technique in future studies.

Acknowledgements

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References

1. Guariguata, L., *et al.* Global estimates of diabetes prevalence for 2013 and projections for 2035. *Diabetes Research and Clinical Practice* **103**, 137-149 (2014).
2. International Diabetes Federation. IDF Diabetes Atlas, 6th edn. (International Diabetes Federation, Brussels, Belgium, 2013).
3. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* **37 Suppl 1**, S81-90 (2014).
4. Nitenberg, A., *et al.* Impairment of coronary vascular reserve and ACh-induced coronary vasodilation in diabetic patients with angiographically normal coronary arteries and normal left ventricular systolic function. *Diabetes* **42**, 1017-1025 (1993).
5. Pitkänen, O.-P., *et al.* Coronary flow reserve is reduced in young men with IDDM. *Diabetes* **47**, 248-254 (1998).
6. Muller, J.M., Davis, M.J. & Chilian, W.M. Integrated regulation of pressure and flow in the coronary microcirculation. *Cardiovascular Research* **32**, 668-678 (1996).
7. Schramm, J.C., Dinh, T. & Veves, A. Microvascular changes in the diabetic foot. *International Journal of Lower Extremity Wounds* **5**, 149-159 (2006).
8. Picchi, A., *et al.* Coronary microvascular dysfunction in diabetes mellitus: A review. *World Journal of Cardiology* **2**, 377-390 (2010).
9. Newman, J.M., *et al.* Norepinephrine and serotonin vasoconstriction in rat hindlimb control different vascular flow routes. *American Journal of Physiology* **270**, E689-699 (1996).
10. Chua, S.C., *et al.* Phenotype of fatty due to Gln269Pro mutation in the leptin receptor (Lepr). *Diabetes* **45**, 1141-1143 (1996).
11. Phillips, M.S., *et al.* Leptin receptor missense mutation in the fatty Zucker rat. *Nature Genetics* **13**, 18-19 (1996).
12. Longair, M.H., Baker, D.A. & Armstrong, J.D. Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics* **27**, 2453-2454 (2011).
13. Doube, M., *et al.* BoneJ: Free and extensible bone image analysis in ImageJ. *Bone* **47**, 1076-1079 (2010).