SGLT2 inhibitor, Canagliflozin, attenuates myocardial infarction in the diabetic and non-diabetic heart

Short title: *SGL2 inhibition attenuates myocardial infarction*

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Abstract

Background:

Recent landmark cardiovascular outcome trials (EMPA-REG OUTCOME and CANVAS) have revealed an unexpected cardiovascular mortality benefit from SGLT2 inhibition in diabetic patients with high cardiovascular risk. We hypothesised that some of this benefit is derived through attenuation of ischemia/reperfusion injury, independent of glycemic status.

Methods and results:

Diabetic and non-diabetic (Zucker Diabetic Fatty [ZDF] and Zucker Lean [ZL]) rats were fed either high-fat or standard chow respectively, with or without fortification with Canagliflozin for 4 weeks. As expected, ZDF rats were markedly diabetic with evidence of end-organ renal injury. ZDF rats responded rapidly to Canagliflozin, significantly lowering serum glucose throughout treatment. In contrast, Canagliflozin had no impact upon serum glucose in ZL rats. After 4 weeks, hearts were harvested, Langendorff-perfused and subjected to 35 minutes regional ischemia and 2 hours reperfusion. Significantly, Canagliflozin pre-treatment led to robust attenuation of infarct size in both diabetic ZDF *and* non-diabetic ZL rat hearts. In contrast, acute treatment with Canagliflozin solubilised in the Langendorff perfusate had no impact upon infarct size.

Conclusion:

This is the first demonstration of a direct cardioprotective effect of an SGLT2 inhibitor in non-diabetic animals against ischemia/reperfusion injury that is independent of an alteration in circulating blood glucose. These data suggest that the benefits of SGLT2 inhibition extend beyond their use in diabetic patients, offering the potential for being re-targeted as a novel cardioprotective therapeutic intervention in high-risk cardiovascular patients irrespective of diabetic status.

Keywords:

Ischemia-reperfusion injury, SGLT2 inhibitor, myocardial infarction, diabetes, cardioprotection

Abbreviations:

EMPA-REG	Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus
CANVAS	CANagliflozin CardioVAScular Assessment Study
DMSO	Dimethyl sulfoxide
GLUT	Glucose Transporter
NHE	Sodium Hydrogen Exchange
NS	Not significant
SGLT1	Sodium Glucose Transporter 1
SGLT2	Sodium Glucose Transporter 2
ZDF	Zucker Diabetic Fatty rat
ZL	Zucker Lean (non-diabetic) rat

Translational Perspective:

SGLT2 inhibitors are known to improve cardiovascular outcomes in high-risk diabetic patients. We demonstrate for the first time that SGLT2 inhibitors attenuate infarct size in both diabetic and non-diabetic rats. This class of anti-hyperglycemic drug therefore appear to have cardioprotective properties that extend beyond their ability to lower circulating blood glucose.

Translational Outlook 1: long-term SGLT2 inhibition is cardioprotective, reducing myocardial infarct size following injurious myocardial ischemia. This is a favourable characteristic for a diabetic therapy, supporting their use in diabetic patients with high risk of, or established, cardiovascular disease.

Translational Outlook 2: our data suggest that infarct limitation is also seen in non-diabetic animals, raising the tantalising potential for re-purposing these drugs to improve cardiovascular outcomes in all high-risk cardiovascular patients, irrespective of diabetic status.

Background

The remarkable cardiovascular benefits of sodium/glucose co-transporter-2 (SGLT2) inhibitors are now well recognised in high-risk type 2 diabetic patients following the landmark clinical trials, EMPA-REG OUTCOME(1) and CANVAS(2). These studies, both designed as non-inferiority investigations mandated by the regulatory authorities, revealed an unexpected benefit and superiority over existing standard diabetic care, with a significant reduction of cardiovascular mortality. Equally remarkably, this reduction in cardiovascular mortality was seen notably early – within 1-2 months – following the introduction of the respective SGLT2 inhibitor. The mechanism underlying the reduction in cardiovascular mortality is not clear and has been subject to much conjecture: seemingly, improvements in blood sugar control were comparatively minor and improvements in terms of diuresis, weight loss and blood pressure reduction inadequate to fully explain the differences observed. Indeed, many, including ourselves, have speculated a potential pleiotropic beneficial effect for this class of glucose-lowering therapy(3-5).

The hypothesis that SGLT2 inhibitors may have pleiotropic effects appears to be supported by other observations from the clinical trial data, not least that SGLT2 inhibition appears to have minimal impact upon the cardiovascular event rate – be it myocardial infarction or stroke, admissions with unstable angina or the need for a coronary revascularisation procedure(1,2). As such, there appears to be minimal impact of SGLT2 inhibition upon macrovascular (arterial atheromatous) disease – but overall, despite suffering the same frequency of cardiovascular events, survival nonetheless appears to be better in those taking SGLT2 inhibitors, a benefit that strikingly manifests within the first few months of treatment.

Cellular injury, necrosis and programmed cell death (apoptosis, necroptosis, autophagy) are important pathophysiological features of a number of maladaptive processes in the heart, including myocardial ischemia and heart failure(6). We therefore hypothesised that despite a similar cardiovascular event rate from events such as acute myocardial ischemia, the improved cardiovascular survival arising from SGLT2 inhibition was through direct myocardial cytoprotection. In a rat, this can be tested in an experimental model of injurious ischemia/reperfusion injury, whereby diabetic animals treated with an SGLT2 inhibitor would be anticipated to have smaller myocardial infarcts. Moreover, if the cardiovascular benefits of SGLT inhibitors are genuinely pleiotropic, we hypothesised that the benefits of SGLT2 inhibition would also be found in those without diabetes.

In designing our experiments, we observed that while the survival curves in EMPA-REG and CANVAS separate quickly, it still takes weeks to see the survival curves diverge. As such, we undertook to "chronically" treat both diabetic and non-diabetic rats for a period of 4 weeks. Moreover, because treatment with an SGLT2 inhibitor will invariably affect circulating blood glucose at the time of myocardial infarction in-vivo, we harvested the hearts and undertook the experiments in an ex-vivo Langendorff model, with perfused glucose concentration controlled in all experiments.

Finally, we wished to ascertain whether the SGLT2 inhibitor would have a direct, cardioprotective effect in the isolated heart, and to this end, we undertook a further group of experiments with "acute" exposure to the SGLT2 inhibitor, with the drug added to the Langendorff perfusate throughout the perfusion protocol.

Using the SGLT2 inhibitor, Canagliflozin, in a reverse-translational study, we found that chronic pre-administration over 4 weeks led to a significant attenuation of myocardial infarct size in **both** diabetic Zucker Diabetic Fatty and non-diabetic Zucker Lean rats. This observation may have significant impact for future translational studies in the repurposing of this new class of glucose-lowering drugs in all patients, irrespective of diabetic status, with high-risk cardiovascular disease.

Methods

For a detailed description of all methods, see the Supplementary material online. In brief, Zucker Lean (ZL) and Zucker Diabetic Fatty (ZDF) were monitored weekly with random blood glucose assessment, and fed either standard or high-fat chow, either fortified with Canagliflozin or without (control) for a period of 4 weeks prior to harvesting the heart and Langendorff perfusion. All feeds, both with and without drug, were prepared by Research Diets Inc. (NJ, USA) based on the diet formulation provided by Janssen Research and Development, Springhouse, PA, USA. Using this formulation, the Canagliflozin-fortified feed results in a circulating Canagliflozin concentration (10 μmol/L) equivalent to that found in human subjects taking maintenance Canagliflozin, 300mg daily(7).

Animals used for the acute administration of Canagliflozin were non-diabetic Sprague-Dawley rats where Canagliflozin (Janssen Research and Development, Springhouse, PA, USA) or vehicle, Dimethyl sulfoxide (0.05% DMSO, Sigma Aldrich, Poole, UK) was perfused throughout the Langendorff experiment.

Randomisation

All experiments were block randomised. Analysis was performed by two blind observers and arbitrated by a third independent adjudicator if required. Once all results were available, the data were un-blinded and analysed.

Statistical analysis

All analyses were performed using GraphPad Prism® version 6 (GraphPad Software, San Diego, CA, USA). The specific statistical test used is reported next to each result. An unpaired t-test was used for two independent groups of continuous variables and a one-way analysis of variance (ANOVA) with Tukey's multiple comparison test for three or more independent groups. Data is presented as mean ± standard error of the mean (SEM). N values are either displayed in the figure or described in the figure legend for each experiment. A significance level of 5% ($\alpha=0.05$) and 80% power ($\beta=0.2$) were used. Statistical significance was reported if $p<0.05$ and results where $p>0.05$ were reported as non- significant (NS).

Results

Characterisation of the ZDF diabetic phenotype

To ensure that our ZDF represented a reasonable facsimile of the diabetic cohort represented within the EMPA-REG and CANVAS studies, we undertook characterisation of the non-diabetic ZL and diabetic ZDF rats. We found, as expected, that the ZDF rats were obese and hyperglycemic (figure 1A, 1B) and hyperglucosuric (figure 1C). In addition, the ZDF rats were found to have evidence of end-organ manifestations of their diabetes, as represented by abnormal renal function and albuminuria (figures 1C and 1D). We are therefore confident that the ZDF represents a reasonable approximation of the human obese type 2 diabetic phenotype with significant and established diabetes at the time of experimentation.

Unexpectedly, we found that diabetic rats treated with Canagliflozin were heavier than untreated diabetics; the expected weight loss from the calorific depletion associated with SGLT2-dependent glycosuria was however observed in the Canagliflozin-treated non-diabetic ZL rats. Growth curves are shown in supplemental section 2.1: the control-diet diabetic ZDF rats started heavier than the non-diabetic ZL rats, but failed to gain significant weight over the 4-weeks of feeding. In contrast, non-diabetic ZL rats gained weight in a linear fashion over the same 4-week period. Interestingly, the pattern and rate of weight gain seen in non-diabetic rats were mirrored in diabetic ZDF rats fed with Canagliflozin, suggesting a healthier animal concomitant with better-controlled diabetes, an interpretation fitting with empirical observations of these animals' physical condition.

Characterisation of the efficacy of Canagliflozin in lowering circulating glucose

To ensure that oral administration of Canagliflozin, via fortification of the chow, was an effective anti-hyperglycemic intervention in our rat model, we observed the random glucose profile in both non-diabetic ZL and diabetic ZDF rats throughout the treatment lead-in period. We found that Canagliflozin was highly effective in lowering blood glucose concentrations in the ZDF rats within a short period from the onset of oral drug administration. Significantly improved blood glucose control was evident throughout the Canagliflozin treatment course compared versus control, with random blood glucose of 16±4 versus 29±1mmol/L respectively ($p=0.002$, figure 2A).

Importantly, Canagliflozin had no impact upon circulating glucose in the non-diabetic ZL rats, with equivalent blood glucose being recorded in both groups (p=NS, figure 2A). Importantly,

we found no evidence of hypoglycemia in either canagliflozin treatment group, despite the presence of significant glucosuria in the Canagliflozin-treated non-diabetic ZL rats (figure 1C).

Interestingly, there was no attenuation of renal dysfunction in the diabetic-Canagliflozin treated group (p=NS, figure 2B, 2C). Unfortunately, our urinalysis assay saturates at glucose levels in excess of 110mmol/L, but higher urinary glucose would be anticipated in this group (figure 1C). In respect to animal mortality, only 2 deaths were recorded – both animals were euthanized for severe urinary tract infection, and these events were found to occur only in animals in the un-treated control diabetic ZDF group (figures 2D and 3).

Impact of 4-week oral Canagliflozin on myocardial infarct size

For this investigation, we used 36 animals. Of these, 9 had to be excluded for reasons summarised in figure 3. 27 animals completed the full experimental protocol.

We found a small but significant difference between myocardial infarct size in the control arms of the diabetic, ZDF and non-diabetic, ZL rat heart groups (p=0.04, figure 4A). This difference is expected in Langendorff-perfused hearts where glucose is the sole energy substrate (see review (8)). We found that Canagliflozin, mirroring the important data by Andreadou *et al* in mouse(9), significantly reduced myocardial infarct size in diabetic ZDF rats. Infarct size relative to the control-chow fed ZDF rats was significantly attenuated, from 37±3% to 20±2% of the area at risk (p=0.001, figure 4A). Importantly, Canagliflozin also significantly abrogated myocardial injury in the non-diabetic ZL rats, reducing infarct size from 55±7% to 27±3% (p=0.001, figure 4A). The area at risk in all control and treatment groups were similar with no statistical difference (figure 4B).

Effect of acute administration of Canagliflozin at the time of ischemia/reperfusion injury

To ascertain whether acute administration of Canagliflozin is protective against injurious ischemia/ reperfusion injury in the non-diabetic rat, we subjected isolated Sprague-Dawley rat heart to ischemia/reperfusion injury in the presence of vehicle (0.05% DMSO) or 10 μmol/L Canagliflozin throughout the perfusion protocol (during 40-minute stabilisation, 35-minute regional ischemia and throughout the 2 hours of reperfusion). The concentration used is equivalent to the plasma concentration of Canagliflozin in diet-fed ZDF rats(7). Baseline characteristics were identical between groups: both demonstrating a non-diabetic level of random blood glucose and identical anthropological measurements between groups (supplemental results table R1). No rats had to be excluded from this study, and all rat data were included in the final analysis.

Of note, acute, ex-vivo Canagliflozin failed to significantly alter infarct size with treatment versus control of 38±3% versus 45±4% respectively (p=0.15, figure 5A). There was no difference in the area at risk between any of the groups (figure 5B).

Discussion

Our study provides the first evidence that long-term oral administration of Canagliflozin over a period of 4-weeks is cardioprotective, ameliorating myocardial infarct size in both diabetic and non-diabetic rats, independent of glucose concentration at the time of ischemia/reperfusion injury. The latter observation, that Canagliflozin-induced protection in the non-diabetic rat, is particularly noteworthy: a clinically-available SGLT2 inhibitor, Canagliflozin, appears to have a cardiovascular and cardioprotective role that extends beyond (and probably also independent of) its intended indication in the management of hyperglycemia in type 2 diabetes mellitus.

Chronic oral Canagliflozin attenuates myocardial infarction in diabetic rat

In the diabetic ZDF rats, attenuating the extent of myocardial necrosis hints towards a novel mechanism underlying the significant reduction of cardiovascular mortality found in the clinical outcome studies, EMPA-REG and CANVAS(1,2). While the clinical data reveal no evidence that SGLT2 inhibitors reduce the number of cardiovascular events such as acute coronary syndromes, they may reduce the myocardial injury that occurs as a consequence of these events. A reduction of myocardial necrosis may thus improve both the immediate and long-term survivability of acute myocardial infarction and reduce the progression into ischemic cardiomyopathy and heart failure – a hypothesis that warrants further investigation.

Interestingly, the protection from chronic ingestion of Canagliflozin was found in hearts that were removed and perfused, ex-vivo, with a perfusate that contained a fixed concentration of glucose (11mmol/L). We designed the experiments this way intentionally to avoid potential confounding effects of glucose-lowering by Canagliflozin at the time of ischemia/reperfusion injury. Moreover, Langendorff-perfusion removes, through washout, other metabolic substrates that may confound Camagliflozin administration (e.g. hepatic generation of ketones, as discussed further below) are excluded as a potential mechanism of cardioprotection. Moreover, that these explanted hearts were protected, despite 40 minutes of crystalloid washout prior to ischaemia, suggests a mechanism that imbues a "memory", potentially through the recruitment of signalling pathways. And if a signalling pathway, it is a pathway who's efficacy, unlike that of ischemic conditioning(11), is seemingly not impacted by the presence of significant diabetes (the severity of the diabetic phenotype confirmed by evidence of the development of nephropathy). One such mechanism may be through a Jak-STAT3 pathway, as suggested by Iliodromitis's group(9) – but there may be others.

Chronic oral Canagliflozin attenuates myocardial infarction in non-diabetic rat

While the observation that Canagliflozin attenuates infarct size in the diabetic rat is important, the principle novelty in this study pertains from our data in the non-diabetic group of animals. We observe that chronic oral Canagliflozin administration significantly reduces myocardial infarct size in non-diabetic ZL rat heart. These data have three provocative implications:

(1) The potentially paradigm-shifting observation that SGLT2 inhibitors may be re-purposed for the management of high-risk **non-diabetic** patients with significant pre-existing cardiovascular disease.

(2) Canagliflozin is not a pure diabetic drug, and possess pleiotropic effects that extend beyond purely lowering serum glucose.

(3) The cardioprotective effect of Canagliflozin is only manifest when administered orally over a period of weeks challenges current thinking in terms of mechanisms that appear to extend beyond a direct effect upon either the myocardium or kidney.

Acute, ex-vivo Canagliflozin fails to protect the non-diabetic rat heart

In contrast to the chronic oral administration, the acute administration of Canagliflozin, ex-vivo, administered at a concentration of 10 μmol/L (equivalent to the circulating concentration in patients taking Canagliflozin, 300mg once daily(7)) throughout the perfusion protocol, failed to reduce infarct size. This concentration of Canagliflozin is also equivalent to rat steady-state circulating serum Canagliflozin concentration from oral digestion of drug, and a concentration that is sufficient to inhibit both SGLT2 and SGLT1, but insufficient to abrogate GLUT activity(12). The observation that acute ex-vivo administration of Canagliflozin fails to protect the isolated heart may provide some further clues to the potential mechanism of action, as it appears to preclude a *direct-acting* cardioprotective effect of the drug upon the myocardium. Administering the drug *ex-vivo* removes any confounding endocrine effects that the drug might elicit from any other organ system *in-vivo*, as might occur in our chronic administration model. Thus, in the absence of infarct attenuation from acute *ex-vivo* administration of Canagliflozin, it would appear that the cardioprotective effect of SGLT inhibition is unlikely to be through the drug acting directly upon the myocardium itself and hints towards an endocrine (and downstream signalling) or metabolic effect to explain the beneficial effect of chronic oral administration of Canagliflozin. However, our data appears not to support a metabolic effect: in our chronic Canagliflozin model, the protection was seen *ex-vivo* with a sole metabolic substrate: glucose at a concentration of 11mmol/L. This makes preferential energy-substrate

switching, as proposed in the ketone hypothesis(10), unlikely as an explanation for the cardioprotection observed. Following explantation and Langendorff-perfusion of the heart, ketones will be rapidly washed out of the coronary circulation as the crystalloid-perfused Langendorff model is associated with far higher coronary flows than found in-vivo(13). Thus, ketones will rapidly fall to negligible levels within the myocardium, and are unlikely to supplant the plentiful supply of glucose as the heart's primary fuel source in the Langendorff perfused model. Of course, we have not excluded the role of endogenous myocardial glycogenesis, but interestingly, chronic SGLT2 inhibition leads to diminution of kidney and liver glycogen stores.(14) The role of glycogen in myocardial ischemia reperfusion injury is complex – canonical succinate synthesis through gluconeogenesis during myocardial ischemia is likely beneficial, but potentially deleterious during reperfusion through reversal of complex II of the mitochondrial transport chain.(15) The impact of glycogen depletion on myocardial injury would be interesting to study further.

The Sodium Hydrogen Exchange (NHE) hypothesis appeared to be a strong and attractive contender to explain the cardioprotection in our chronic Canagliflozin administration studies(16,17). Previous investigations using Cariporide and Amiloride in animal models reveal highly efficacious anti-ischemic benefits of NHE inhibition against myocardial infarction, particularly when administered prior to the onset of myocardial ischemia(18-21). Thus, we had anticipated the acute ex-vivo study to provide further evidence of infarct size limitation. Indeed, in the excellent study from Zuurbier's group, with 3 μmol/L Canagliflozin[17], they demonstrated highly effective attenuation of NHE activity. Given the similarity in concentration of Canagliflozin in our and in Zuurbier's cell-based model, we were surprised that we found no protection in our acute model. Might the protective effects of chronic administration of Canagliflozin be mediated through NHE inhibition? Encouragingly, protection was observed in both diabetic and non-diabetic animals as expected. However, with 40 minutes of washout prior to induction of ischaemia, it seems somewhat unlikely that significant quantities of Canagliflozin would remain within the heart. Our data would therefore appear to suggest that the observed protection from chronic administration of Canagliflozin is less likely to be mediated through NHE inhibition, but perhaps through another pleiotropic pathway capable of triggering a "memory" effect through activation of signalling cascades. Already identified candidate pathways include the aforementioned Jak/STAT3 pathway(9) that may also help attenuate oxidative stress and fibrotic myocardial remodelling(22) or perhaps

through AMPK(23) (also found in kidney to reduce ischemia/ reperfusion injury(24)), although these are not hypotheses that we have yet tested.

Finally, SGLT2 inhibitors have been found to imbue significant protection in the vasculature of diabetic ZDF rats, with preservation of endothelial function. This endothelial protection appears to be mediated through attenuation of chronic glucotoxicity and amelioration of oxidative stress.(25) This could translate into myocardial protection ex-vivo, but we did not find significant differences in coronary flow in our model between Canagliflozin-treated verses control treated animals (data shown in supplemental section 2.5). Moreover, if the protection were mediated primarily as a mechanism designed to abrogate glucotoxicity, this hypothesis fails to explain why Canagliflozin protects the non-diabetic heart. However, it would be interesting to repeat these experiments in the non-diabetic Zucker Lean to see whether the cytoptotective phenotype is evident in the absence of injurious elevated blood glucose.

Canagliflozin mediated cardioprotection appears independent of circulating glucose

As expected, we found Canagliflozin to be highly effective at reducing circulating blood glucose in our diabetic rat model. While we did not see the random blood glucose level in Canagliflozin-treated diabetic ZDF rats fall into the non-diabetic range, the drug was nonetheless still highly effective at reducing infarct size, suggesting that complete restoration of random blood glucose into the “normal” non-diabetic range is unnecessary to imbue the cardioprotection observed. Moreover, Canagliflozin failed to impact on circulating blood glucose levels in the non-diabetic animals: random glucose levels were identical in both non-diabetic control and Canagliflozin-treated rats. There are two observations in respect to this data: (1) that Canagliflozin can be administered to non-diabetic animals without fear of triggering potentially injurious hypoglycemia and (2) that lowering blood glucose is not a prerequisite for attenuation of myocardial infarct size. Therefore, glucose lowering in the diabetic ZDF animals is a good biomarker of Canagliflozin-mediated SGLT2 inhibition, but the in-vivo lowering of glucose is not conditional for the triggering of infarct-size reduction when the heart is explanted and perfused ex-vivo. Furthermore, as alluded to above, as the hearts were maintained with a perfused glucose concentration of 11mmol/L throughout perfusion, any confounding effect of differences in circulating glucose concentration is effectively removed from our experiment.

Finally, it is also interesting to observe that chronic oral Canagliflozin is equally protective in both non-diabetic and diabetic animals. This contrasts with the majority of cardioprotective

interventions whose efficacy are blunted in the presence of the diabetic phenotype(11). This therefore leads us to speculate that the mechanisms of protection are different from, and potentially additive to, more established experimental models of myocardial protection, such as ischemic or pharmacological conditioning. If this were to be the case, then it offers the opportunity to augment myocardial protection through combined therapeutic approaches at the time of presentation of an acute coronary syndrome, to optimise patient outcome.

Absence of reno-preservation

In establishing our diabetic model, we wanted to determine the severity of the diabetic phenotype. The SGLT2 outcome studies have all been performed in models of established type 2 diabetes mellitus, and typically in patients with high cardiovascular risk. We therefore wanted to ascertain whether our model displayed characteristics of diabetic end-organ damage in the form of albuminuria. Our diabetic ZDF rats did indeed display evidence of significant albuminuria at the point at which the hearts were harvested for ex-vivo Langendorff perfusion. The lack of any meaningful difference between the Canagliflozin-fed and control ZDF rats is not however unexpected. The reno-protective effects of SGLT2 inhibition typically take many months to manifest(2,26), which contrasts with the comparatively rapid separation of the cardiovascular outcome curves. We designed our study primarily as an investigation into cardioprotection; a study with renoprotection as a primary end-point would likely mandate a much longer duration of drug treatment.

Diabetic complications

It was initially surprising that the only serious, life-threatening complication found during our chronic study was infective. As might have been anticipated, the source of infection was, in both cases, urinary tract. However, these two events were in the non-treated control diabetic ZDF rats and not in animals treated with Canagliflozin. In total, two animals in control ZDF group had to be euthanized for serious sepsis; neither of the Canagliflozin treated groups (diabetic or non-diabetic) had evidence of septic complications. Both diabetic ZDF groups had significant glycosuria, whereas the untreated control ZDF also suffered with significant hyperglycemia. The sepsis therefore is much more likely to be secondary to the uncontrolled diabetes in the control animals, whereas the infective risk associated with Canagliflozin-induced glucosuria was easily managed by simple animal husbandry and hygiene methods. No animal deaths were found related to cardiovascular causes, but our study was not powered for

this endpoint, and nor was it run for a sufficient period for such complications to become manifest.

Conclusions

We demonstrate that chronic oral administration of Canagliflozin results in significant reduction in myocardial infarct size, irrespective of glucose lowering or the presence of diabetes. This protection appears not to be mediated via a direct effect of Canagliflozin upon the myocardium, but via an intermediate signalling mechanism that has yet to be identified. Our study therefore provides new insights into the potential cardiovascular benefits of SGLT2 inhibition and even points to a potential and important translational re-purposing of these drugs to reduce cardiovascular mortality in non-diabetic patients.

Study limitations

In designing our studies, we accepted a number of compromises. To avoid the confusion that may ensue with polypharmacy, we did not treat the control diabetic animals to manage their hyperglycemia. These animals displayed high levels of glycemia, and two animals suffered with septic complications that were rapidly identified and managed. We therefore feel that prolonging the duration of study beyond four weeks as designed would not have been feasible. However, the infarct size data is compelling: administering Canagliflozin, irrespective of diabetic status, resulted in a pronounced reduction of myocardial infarct size.

As all diabetic patients in the clinical outcome studies were undertaken in the presence of anti-hyperglycemics, a future study may be constructed at the outset to include diabetic animals managed with metformin, the backbone of contemporary type 2 diabetic management. Indeed, this may well be mandated in any future study designed to look at cardiovascular complications and renal outcomes where much longer treatment periods would need to be considered.

We do not believe that the severity of the diabetes impacted adversely upon the outcome of our study; in fact, the infarct size of the diabetic animals was entirely in-line with previous short-term studies in other diabetic models (such as streptozocin-treated or Goto-Kakizaki lean diabetic) and from our own group and others(27,28). However, having established that Canagliflozin is cardioprotective, it would be useful to demonstrate that this protective phenotype is reproducible on top of existing strategies for managing elevated blood sugar.

Interestingly, it is well recognised that diabetic hearts, when Langendorff-perfused with glucose with as the sole substrate, will have a smaller infarct size compared to the non-diabetic

heart under the same conditions (see review(8)). While a reductionist approach in metabolic substrate provision has its limitations, there are advantages in that we have excluded other potential metabolic substrates that have been postulated (such as ketone bodies). From our data, future more in-depth analysis of the myocardial metabolome may be undertaken, and, for example, the impact of any glycogen depletion that may result from chronic SGLT2 inhibition, investigated.

Finally, our acute Canagliflozin study was performed in Sprague Dawley rats, rather than the Zucker Lean strain. Neither strain of rat are diabetic. Both strains reveal similar infarct sizes when subjected to 35 minutes of regional ischemia and 2 hours reperfusion. While there are differences between individual strains of murine and rat models and their sensitivity to myocardial ischemia/reperfusion injury, given baseline similarities in infarct size, we would have expected Canagliflozin to be as protective in Sprague Dawley rats as the Zucker Lean. The absence of protection observed is therefore informative, but minor strain differences cannot be completely excluded.

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Conflict of interest: none declared

Figure legends

Figure 1: Characterisation of the ZL and ZDF phenotypes. **Panel A:** body weight index. The diabetic ZDF rats were significantly larger than the non-diabetic ZL rats. Canagliflozin administration in the ZL led to a significant reduction in body mass index that was absent in the ZDF diabetic rats. n=8-10 per group. **Panel B:** random glucose concentration on day of experiment. As expected, ZDF diabetic rats had significantly higher blood glucose concentrations compared to the non-diabetic ZL controls ($p<0.0001$, n=6-9 per group). Canagliflozin had no impact upon blood glucose in the ZL group (p=NS, n=9-10 per group), but significantly reduced glucose in the diabetic ZDF rats ($p<0.0001$, n=6-9 per group). Renal manifestations of diabetes in the ZDF rats: panels C to E. **Panel C:** urine glucose, measured by urinalysis strip test. No glucosuria was detectable in the control ZL rats, but significant glucosuria in ZL rats on Canagliflozin. As expected, significant glucosuria was found in both ZDF control and Canagliflozin-treated groups. **Panel D:** blood urea nitrogen (BUN) was significantly higher in the ZDF rats compared to the non-diabetic ZL: 11±2 versus 19±2 mg/dL ($p=0.006$, n=6 per group). **Panel E:** a similar pattern was observed in the urine albumin:creatinine ratio – the diabetic ZDF rats demonstrating a significantly higher albumin excretion compared to the non-diabetic ZL rat: 160±39 versus 3319±577 mg/g ($p=0.0004$, n=4-5 per group).

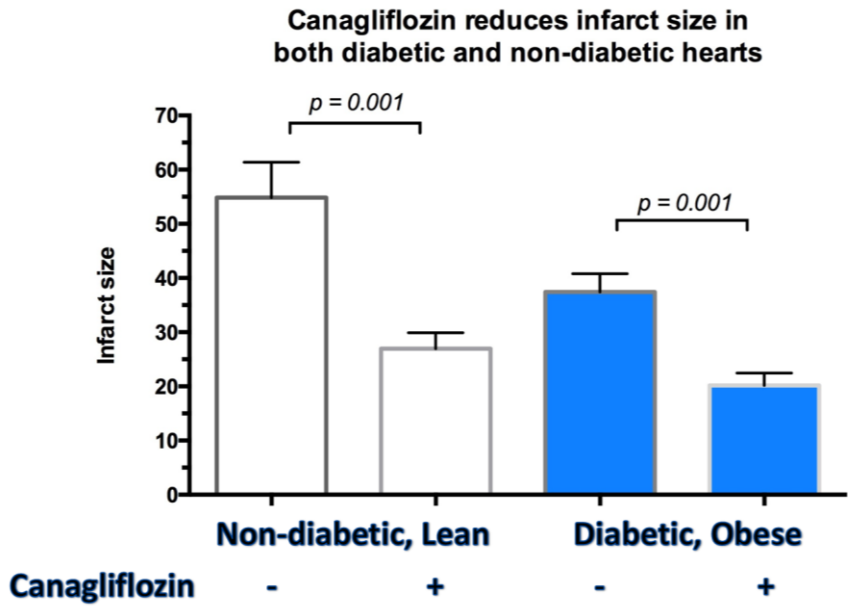
Figure 2: Impact of Canagliflozin in non-diabetic and diabetic ZL and ZDF rats. **Panel A:** Canagliflozin had a rapid and sustained impact upon circulating blood glucose in the diabetic ZDF rats compared to the untreated animals. In contrast, Canagliflozin had no impact upon circulating blood glucose in the non-diabetic ZL rats ($p=0.002$, n=8-10 per group). **Panel B:** After 4-weeks treatment, Canagliflozin had little impact upon blood urea nitrogen in either ZL or ZDF rats (p=NS, n=6-8 per group). **Panel C:** As with BUN, there was little impact from 4-week oral Canagliflozin administration in either ZL or ZDF rats upon albumin:creatinine ratios (p=NS, n=4-8 per group). **Panel D:** Kaplan-Meier survival curve. Two animals, both in the control diabetic ZDF group, had to be euthanised for severe urinary sepsis. All other groups completed without events.

Figure 3: CONSORT-style diagram for Infarct Assessment in the Chronic-Administration Study. 36 animals were started into the study, of which 29 completed through to analysis. Reasons for and timings of animal exclusions shown in all groups. *Pre-priori* exclusion criteria are shown in Supplemental material (Appendix 1).

Figure 4: Infarct size reduction following chronic 4-week oral administration of Canagliflozin. **Panel A:** In both diabetic ZDF and non-diabetic ZL rats, we found a significant reduction of infarct size compared to control. In non-diabetic rats, infarct size was reduced from 55±7 to 27±3% (p=0.001, n=6-8 per group). In the diabetic ZDF rats, a similar reduction of infarct size was also observed with infarct size reducing from 37±3 to 20±2% (p=0.001, n=6-8). There was a modest but significant difference in infarct size between control-diet treated ZL and ZDF rats (p=0.04). **Panel B:** area at risk in all groups were equivalent (p=NS, n=6-8 per group).

Figure 5: Infarct sizes following acute ex-vivo administration in non-diabetic Sprague-Dawley rats. **Panel A:** in contrast to the cardioprotective effect of 4-week oral administration of Canagliflozin, we found no evidence of infarct reduction with acute, ex-vivo administration of Canagliflozin, infarct sizes of 45±4 versus 38±3% (p=NS, n=6 per group) in the vehicle control group. **Panel B:** There was no difference in the area at risk in either of the treatment groups (DMSO vehicle control versus Canagliflozin, p=NS, n=6 per group).

Central Illustration



Above: Canagliflozin, at a clinically-relevant dose, reduces myocardial infarct size following injurious myocardial ischemia and reperfusion in both diabetic *and* non-diabetic hearts (n=6-8 per group).

One sentence Summary

Canagliflozin, an SGLT2 inhibitor, significantly attenuates myocardial infarct size in both diabetic **and** non-diabetic heart, potentially pointing the way to re-purposing SGLT2 inhibitors in **all** high cardiovascular risk patients irrespective of their diabetic status.

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