Glucose inhibits neutrophil migration and haemostasis, and accelerates diet-induced hyperlipidaemia in zebrafish embryos

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ABSTRACT

Hyperglycaemia damages the microvasculature in part through the reduced recruitment of immune cells and interference with platelet signalling, leading to poor wound healing and accelerated lipid deposition in mammals. We investigated the utility of zebrafish embryos to model the effect of glucose on leukocyte recruitment, thrombosis, and hyperlipidaemia by treating embryos with exogenous glucose. Exogenous glucose supplementation reduced neutrophil recruitment, and thrombocyte and fibrin plug formation following tail transection in zebrafish embryos, but did not affect the recruitment of macrophages. We observed accelerated lipid accumulation in glucose-supplemented embryos challenged with high fat chicken egg yolk feeding. Our study identifies conserved and divergent effects of high glucose in the tractable zebrafish embryo model system compared to mammals.

INTRODUCTION

Hyperglycaemic damage to the microvasculature is hypothesised to underpin much of the pathology associated with diabetes in mammals, including perturbations to leukocyte biology, haemostasis, and the accumulation of lipid laden macrophages in the vessel wall [1-4]. Previous mammalian research has demonstrated that hyperglycaemia damages the microvasculature resulting in reduced expression of endothelial adhesion molecules for immune cell recruitment [2, 4]. As a result, fewer neutrophils and macrophages are recruited to diabetic wounds [1, 2, 5]. Of the macrophages and neutrophils that eventually arrive, there is a skewing of differentiation towards an inflammatory phenotype owing to the inflammatory nature of the diabetic wound microenvironment [6, 7].

Mammals with hyperglycaemia demonstrate perturbed coagulation and platelet signalling, causing disruption of haemostasis [3, 8]. Reduced efficiency of haemostasis results in unstable and ineffective clots within diabetic foot ulcers [3, 9]. Treatments for diabetic foot ulcers can involve platelet and fibrin therapy, indicating an important role for inadequate fibrin clot production in the ulceration process [10, 11].

Hyperglycaemia-induced damage to the microvasculature also increases vascular lipid accumulation in conjunction with hyperlipidaemia [12]. Hyperlipidaemia and hyperglycaemia are compounding risk factors for the development of Type 2 diabetes, associated with the ‘Western Diet’ consisting of fat and sugar alongside limited exercise [13, 14]. There are multiple mechanisms by which hyperglycaemia damages the microvasculature: through the formation of advanced glycation products [3], the induction of oxidative stress [15], interfering with nitric oxide production [16], and inducing macrophages to form lipid-laden foam cells [17]. The degradation of the endothelial structural integrity increases the rate of lipid deposition by providing a physical niche for lipid infiltration [12]. This can ultimately lead to the development of atheroma, which are common in diabetic patients [17].

Our study investigates the effect of high glucose environments on inflammation, thrombosis and hyperlipidaemia using a zebrafish model. Zebrafish are established models for investigating each of these processes in isolation [18-21]. Pertinent to this study, zebrafish have similar clotting, metabolic, and immune systems to mammals, and these have been used to provide insight into the shared function of these systems across vertebrates [19, 20, 22]. Here we have combined these models to investigate the role of high glucose environments in disrupting immune cell recruitment to wounds, thrombosis, and lipid accumulation in zebrafish embryos.

METHODS

Zebrafish Husbandry

Zebrafish embryos were produced through natural spawning (Sydney Local Health District Animal Welfare Committee Approval 17-036). The strains used were *Tg(lyzC:DsRed)*^{nz50} to visualise neutrophils, *Tg(mfap4:turquoise)*^{xt27} to visualise macrophages, *Tg(itga2b:gfp)*^{la2} to visualise thrombocytes, and *Tg(fabp10a:fgb-gfp)*^{mi4001} to visualise fibrin deposition. From 1-5 days post fertilisation (dpf) embryos were kept in a dark incubator at 28°C.

Induction of High Glucose Concentrations in the Zebrafish

Injection method: Eggs were injected with approximately 15 nmol of glucose, or PBS as a control within four hours of fertilisation (Figure 1A). Immersion method: Dechorionated 2 dpf embryos were immersed in 5% mannitol (osmolarity control) or glucose dissolved in E3 media (Figure 1B), media was changed daily to reduce microbial growth.

Glucose Oxidase Assay

Embryos were snap frozen and stored at -20°C. Embryos were lysed in the buffer solution from the Amplex Red Oxidase kit (Sigma: A22188), lysates were sedimented by centrifugation, and the

Amplex Red Glucose oxidase kit was used to measure the glucose content of the supernatant in accordance with the manufacturer's instructions.

Tail Transection Assays

Caudal fin amputations were performed on embryos at 5 dpf. Embryos were anesthetised with 2.5% (v/v) ethyl-3-aminobenzoate methanesulfonate (tricaine) (Sigma, E10521), wounded posterior to the notochord using a sterile scalpel and kept in a 28°C incubator to recover as previously described [23]. Wounded embryos were imaged at 6 hours (neutrophil and macrophages) or 2.5 hours (fibrin and thrombocytes).

Hyperlipidaemia Assay

Post 5 dpf, embryos were transferred to a 28°C incubator with a 14/10 hour light/dark cycle. Embryos were placed in an E3 solution containing 0.05% of emulsified chicken egg yolk from 5 dpf. Each day, a random sample of embryos were removed, euthanised, and fixed in paraformaldehyde. Embryos were stained with Oil Red O to quantitate lipid accumulation, as previously described [21, 24, 25].

Imaging and Image Analysis

Embryos were imaged using a Leica M205FA fluorescent microscope. ImageJ software was used to quantify fluorescent pixel count within 100 µm of the wound site for transgenic wound assays as previously described [23, 26].

Oil Red O staining was quantified in ImageJ by adjusting the colour threshold to eliminate non-red signal. The image was then converted to a binary mask, and the tail region posterior to the swim bladder was selected to measure the number and area of particles [25].

Statistical analysis

Outliers were excluded using a 1% ROUT test. Statistical testing was carried out by ANOVA with correction for multiple comparisons or Student's *t*-tests as appropriate using GraphPad Prism. Data are expressed as mean ± SD. Every datapoint represents a single embryo unless otherwise noted.

RESULTS

Exogenous glucose exposure increases glucose in zebrafish embryos

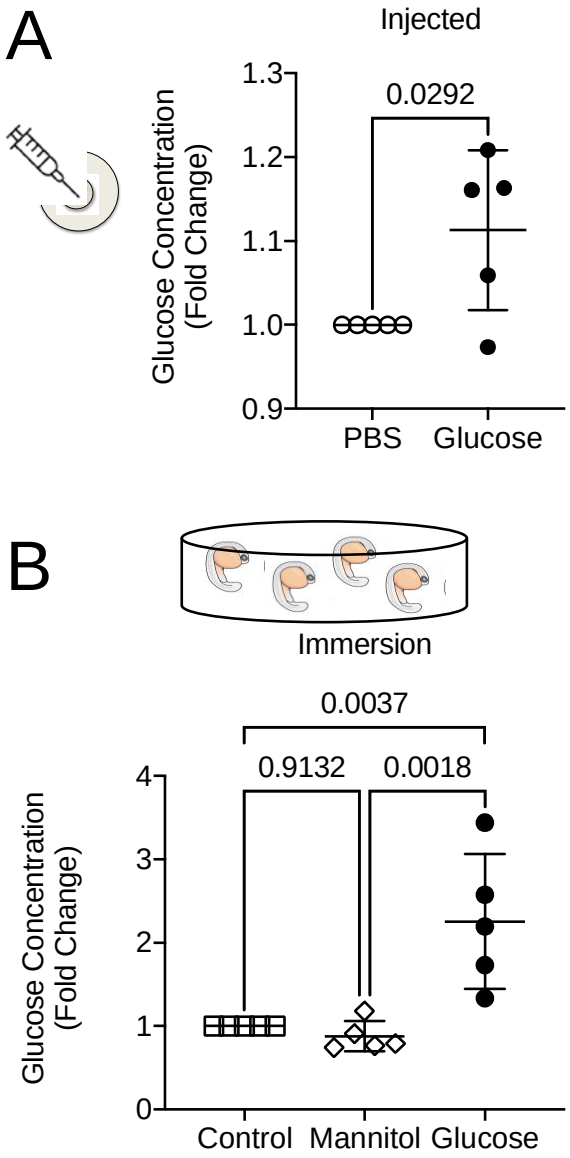

Figure 1: Injection and immersion methods increase glucose levels in zebrafish embryos.

A. Relative concentration of glucose in 5 dpf embryos that had been injected with 15 nmol glucose as eggs. Statistical testing by *t*-test, each data point is representative of a group of n = 10-30 embryos.

B. Relative concentration of glucose in 5 dpf embryos immersed in 5% solutions of glucose or mannitol since 2 dpf. Statistical testing by ANOVA, each data point is representative of a group of n = 10-30 embryos.

To establish the efficacy of the injection and immersion techniques to increase glucose concentrations in 5 dpf zebrafish embryos, we conducted a glucose oxidase assay. Consistent with past literature, we observed an increase in the glucose concentration contained within the glucose-injected and -immersed embryos compared to controls (Figure 1) [27, 28]. The injection method was selected for our study because it decreased the increased risk of microbial overgrowth compared to the immersion method.

Glucose impedes neutrophil, but not macrophage, chemotaxis in zebrafish embryos.

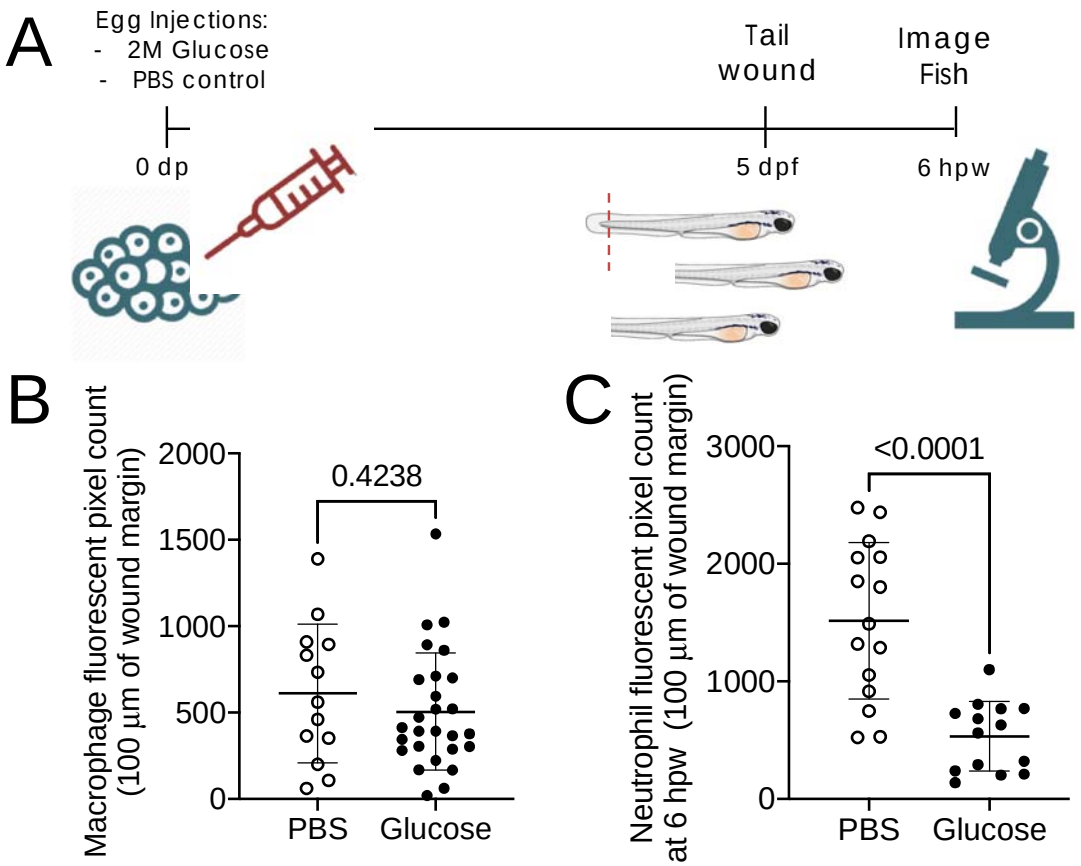

Figure 2: High glucose reduced neutrophil, but not macrophage, recruitment to a tail wound.

A. Schematic of experiment to measure immune cell recruitment to a tail wound.

B. Quantification of macrophage recruitment following tail transection.

C. Quantification of neutrophil recruitment following tail transection.

Statistical testing by *t*-test. Data are from 2 biological replicates.

Altered innate immune cell recruitment to wounds is a conserved feature of hyperglycaemia in mammals [4, 5, 29-31]. To determine if this phenomenon was conserved in zebrafish embryos, we utilised the tail transection wound model which causes reproducible leukocyte recruitment [23] (Figure 2A). We first performed this assay using transgenic *Tg(mfap4:turquoise)*^{xt27} embryos, where macrophages are marked by turquoise fluorescent protein to quantify macrophage recruitment. Surprisingly, we observed no difference in the number of recruited macrophages between the glucose-injected and control embryos at 6 hours post wounding (hpw) (Figure 2B).

We then used *Tg(lyzC:DsRed)*^{nz50} embryos, where neutrophils are marked by DsRed fluorescent protein, to measure the recruitment of neutrophils. We observed a reduction in neutrophil recruitment between the control and glucose-injected embryos at 6 hpw (Figure 1C).

Together, these results indicate that neutrophil, but not macrophage, recruitment is perturbed by high glucose in zebrafish embryos.

Glucose impedes haemostasis in zebrafish embryos

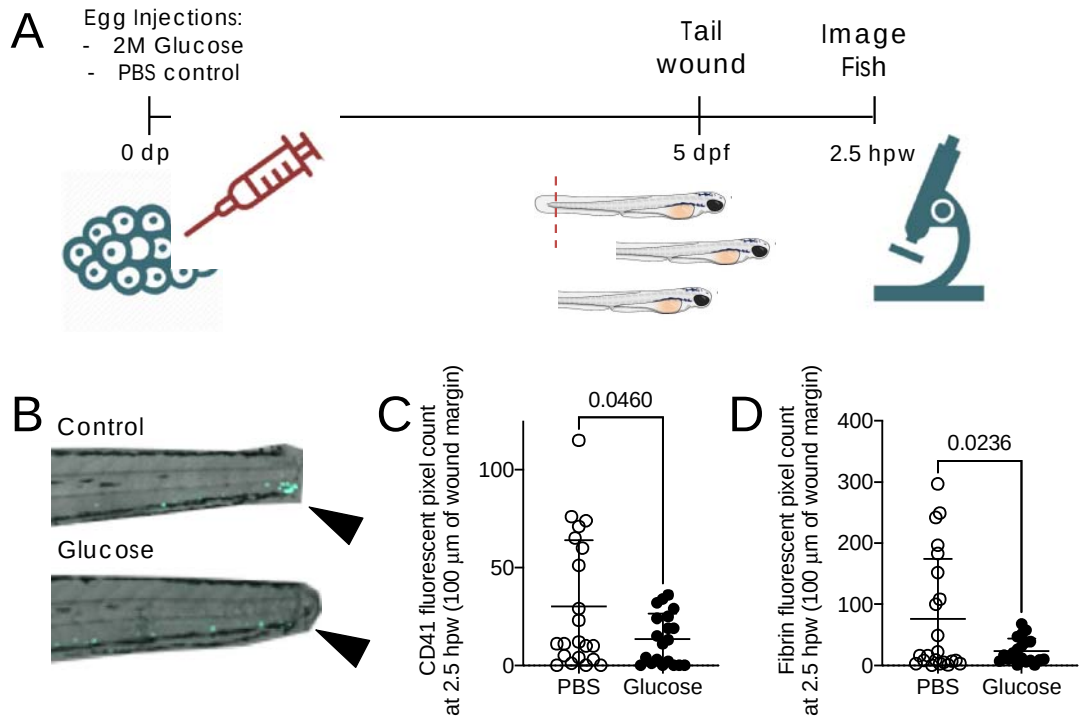

Figure 3: High glucose reduced thrombocyte and fibrin accumulation to a tail wound.

A. Schematic of experiment to visualise haemostasis following tail transection.

B. Representative overlay images of thrombocytes (green) demonstrating recruitment to tail wound in control and glucose *Tg(itga2b:gfp)*^{la2} embryos. Arrowhead indicates location of severed major blood vessels at tail transection site.

C. Quantification of thrombocyte plug size following tail transection.

D. Quantification of fibrin clot size following tail transection.

Statistical testing by *t*-test. Data are representative of 2 biological replicates.

Hyperglycaemia perturbs coagulation and platelet activation in mammals, resulting in ineffective haemostasis [3, 8]. To visualise the effects of high glucose on haemostasis in zebrafish embryos, we first used the *Tg(itga2b:gfp)* ^{la2} line where thrombocytes are tagged with GFP [32, 33]. Following tail transection (Figure 3A), high glucose *Tg(itga2b:gfp)* ^{la2} embryos demonstrated reduced thrombocyte accumulation (Figure 3B-C).

To determine if the reduction in thrombocyte recruitment was mirrored by perturbed clotting, we conducted tail transections on *Tg(fabp10a:fgb-gfp)*^{mi4001} embryos which express fluorescently tagged fibrinogen and allow visualisation of clots [34]. Glucose-injected *Tg(fabp10a:fgb-gfp)*^{mi4001} embryos had reduced fibrin accumulation following tail transection compared to PBS-injected embryos (Figure 3D).

Together, these results demonstrate that a high glucose environment inhibits haemostasis in zebrafish embryos.

High glucose accelerated hyperlipidaemia in zebrafish embryos challenged with a high fat diet

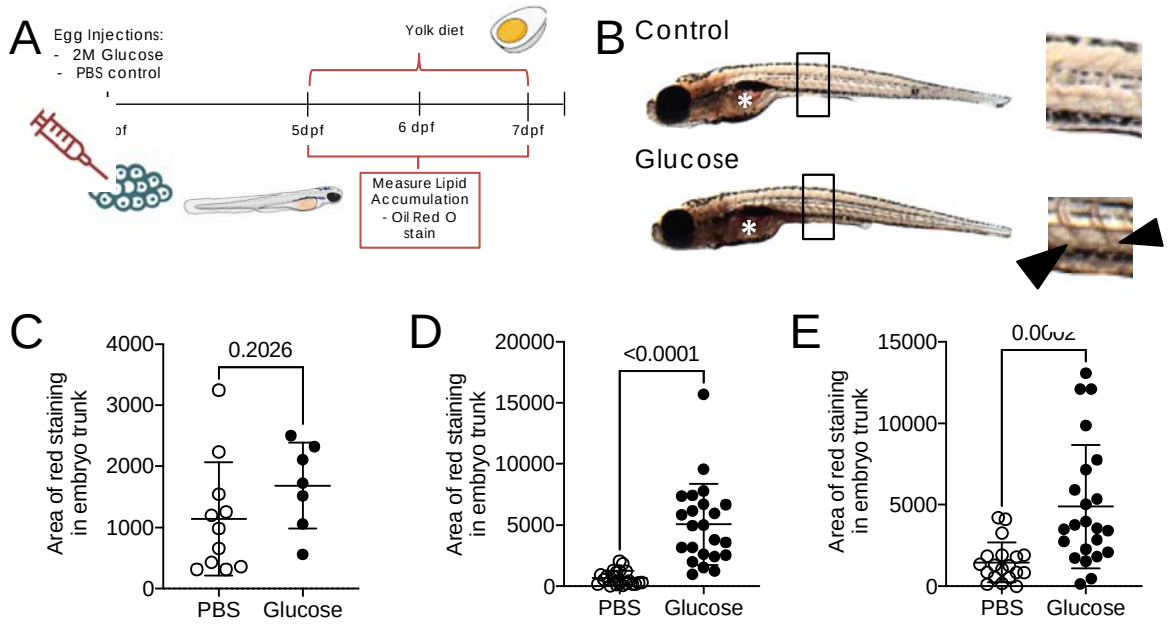

Figure 4: Glucose-injected embryos have increased lipid accumulation following a high fat diet.

A. Schematic of the high fat feeding challenge assay to measure lipid accumulation in glucose-injected embryos.
B. Bright field images of 6 dpf Oil Red O-stained embryos, demonstrating darker vascular staining in glucose-injected embryos. Box indicates location of inset, arrowheads indicate stained intercostal vessels in inset, asterisk indicates intestinal lumen which was excluded from analysis.
C. Quantification of lipid accumulation in 5 dpf glucose-injected embryos.
D. Quantification of lipid accumulation in 6 dpf glucose-injected embryos challenged with a high fat diet from 5 dpf.
E. Quantification of lipid accumulation in 7 dpf glucose-injected embryos challenged with a high fat diet from 5 dpf.

Statistical testing by *t*-test. Data are representative of 2 biological replicates.

Hyperglycaemia and hyperlipidaemia are intimately associated in mammals [35]. To determine if this interaction is conserved in zebrafish embryos, we fed glucose-injected embryos a high fat diet

consisting of emulsified chicken egg yolk from 5-7 dpf (Figure 4A). Glucose- and PBS-injected embryos had similar Oil Red O vascular staining prior to the initiation of chicken egg yolk feeding at 5 dpf (Figure 4B). Quantification of Oil Red O staining revealed glucose-injected embryos had increased vascular lipid content at one and two days post feeding (Figure 4D-E).

These results recapitulate the interaction between hyperglycaemia and hyperlipidaemia seen in mammals [15], demonstrating that zebrafish embryos are a robust model to study this conserved interaction.

DISCUSSION

In this study, we explored the effect of high glucose levels on inflammation, thrombosis, and lipid accumulation in the zebrafish embryo model. We found that high glucose reduces neutrophil recruitment and haemostasis around wound sites. In addition, we determined that high glucose accelerates lipid accumulation when embryos are challenged with a high fat diet.

Hyperglycaemia in mammals causes vascular dysfunction that restricts the recruitment of a broad range of immune cells to wounds [16]. It was therefore surprising that high glucose appeared to have no effect on the recruitment of macrophages to the tail wound in this study. It is possible that high glucose in zebrafish embryos does not affect abluminal crawling of macrophages along blood vessels, since wound-responsive macrophages have been demonstrated to move predominantly through interstitial tissue in zebrafish embryos [5, 36].

Our finding that high glucose environments reduce the recruitment of neutrophils to a tail wound is consistent with studies of diabetic foot ulcers [2, 4]. In mammals, high glucose environments have been shown to interfere with metabolites and enzymes related to neutrophil function [37]. Neutrophils may also be restricted by hyperglycaemia mediated vascular dysfunction as they are

more reliant on extravasation to reach distal sites in zebrafish embryos than macrophages [16, 36]. Although neutrophil dysfunction was conserved, macrophage function was unaffected by high glucose environments suggesting that glucose injection of zebrafish embryos may not be a suitable platform for studying the impact of high glucose on leukocyte recruitment.

The relationship between hyperglycaemia and hyperlipidaemia has been previously reported in various mammalian species [15, 28, 38], and a recent study by Wang *et al*. has demonstrated a similar the interaction of a high cholesterol diet with glucose immersion on vascular lipid accumulation in zebrafish after 10 days of feeding [28]. Our study using chicken egg yolk as a high fat diet challenge demonstrates a dramatically accelerated accumulation of lipid after just one day of feeding. This chicken egg yolk feeding-based model, therefore provides a more rapid model to investigate lipid accumulation.

In summary, we report glucose-supplemented zebrafish embryos as a tractable platform to investigate the conserved interactions between glucose and neutrophil biology, haemostasis, and diet-induced hyperlipidaemia.

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