

1 **Glucose inhibits haemostasis and accelerates diet-induced hyperlipidaemia in zebrafish larvae**

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11

12 **ABSTRACT**

13 Hyperglycaemia damages the microvasculature in part through the reduced recruitment of immune
14 cells and interference with platelet signalling, leading to poor wound healing and accelerated lipid
15 deposition in mammals. We investigated the utility of zebrafish larvae to model the effect of
16 glucose on neutrophil and macrophage recruitment to a tail wound, wound-induced haemostasis,
17 and chicken egg yolk feed challenge-induced hyperlipidaemia by supplementing larvae with
18 exogenous glucose by immersion or injection. Neither method of glucose supplementation affected
19 the recruitment of neutrophils and macrophages following tail transection. Glucose injection
20 reduced thrombocyte retention and fibrin plug formation while only thrombocyte retention was
21 reduced by glucose immersion following tail transection. We observed accelerated lipid
22 accumulation in glucose-injected larvae challenged with high fat chicken egg yolk feeding. Our
23 study identifies conserved and divergent effects of high glucose on inflammation, haemostasis, and
24 hyperlipidaemia in zebrafish larvae compared to mammals.

25

26 **INTRODUCTION**

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

35

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

41

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

52

53 These phenotypes have not been previously investigated in fish. Our study investigates the effect of
54 high glucose environments on inflammation, thrombosis and hyperlipidaemia using a zebrafish
55 model. Zebrafish are established models for investigating each of these processes in isolation¹⁸⁻²¹.
56 Pertinent to this study, zebrafish have similar clotting, metabolic, and immune systems to mammals,
57 and these have been used to provide insight into the shared function of these systems across
58 vertebrates^{19,20,22}. Here we have combined these models to investigate the role of high glucose
59 environments in disrupting thrombosis and lipid accumulation, but not immune cell recruitment to a
60 wound, in zebrafish larvae.

61

62 **METHODS**

63 **Zebrafish Husbandry**

64 Zebrafish embryos were produced through natural spawning (Sydney Local Health District Animal
65 Welfare Committee Approval 17-036). The strains used were *Tg(lyzC:DsRed)^{nz50}* to visualise
66 neutrophils²³, *Tg(mfap4:turquoise)^{xt27}* to visualise macrophages²⁴, *Tg(itga2b:gfp)^{la2}* to visualise
67 thrombocytes²⁵, and *Tg(fabp10a:fgb-gfp)^{mi4001}* to visualise fibrin deposition²⁶. From 1-5 days post
68 fertilisation (dpf) embryos were kept in a dark incubator at 28°C.

69

70 **Induction of High Glucose Concentrations in the Zebrafish**

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

75

76 **Glucose Oxidase Assay**

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

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

81

82 **Tail Transection Assays**

83 Caudal fin amputations were performed on larvae at 5 dpf. Larvae were anaesthetised with 2.5%
84 (v/v) ethyl-3-aminobenzoate methanesulfonate (tricaine) (Sigma, E10521), wounded posterior to
85 the notochord using a sterile scalpel and kept in a 28°C incubator to recover as previously described
86 ²⁷. Wounded larvae were imaged at 6 hours (neutrophil and macrophages) or 2.5 hours (fibrin and
87 thrombocytes).

88

89 **Hyperlipidaemia Assay**

90 Post 5 dpf, larvae were transferred to a 28°C incubator with a 14/10 hour light/dark cycle. Larvae
91 were placed in an E3 solution containing 0.05% of emulsified chicken egg yolk from 5 dpf. Each
92 day, a random sample of larvae were removed, euthanised, and fixed in paraformaldehyde. Larvae
93 were stained with Oil Red O to quantitate lipid accumulation, as previously described ^{21,28,29}.

94

95 **Imaging and Image Analysis**

96 Larvae were imaged using a Leica M205FA fluorescent microscope. ImageJ software was used to
97 quantify fluorescent pixel count within 100 µm of the wound site for transgenic wound assays as
98 previously described ^{27,30}.

99

100 Oil Red O staining was quantified in ImageJ by adjusting the colour threshold to eliminate non-red
101 signal. The image was then converted to a binary mask, and the tail region posterior to the swim
102 bladder was selected to measure the number and area of particles ²⁹.

103

104 **Statistical analysis**

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

108

109 **RESULTS**

110 **Exogenous glucose exposure increases glucose in zebrafish larvae**

111 To establish the efficacy of the injection and immersion techniques to increase glucose
112 concentrations in 5 dpf zebrafish larvae, we conducted a glucose oxidase assay. Consistent with past
113 literature, we observed an increase in the glucose concentration contained within the glucose-
114 injected and -immersed larvae compared to controls (Figure 1A and 1B)^{31,32}.

115

116 Glucose immersion caused sporadic microbial overgrowth and we observed reduced growth of
117 larvae immersed in either glucose or mannitol, but not in glucose-injected larvae, as measured by
118 total body area (Figure 1C and 1D) or eye area (Figure 1E and 1F).

119

120 We next analysed the effect of glucose supplementation on the development of key innate immune
121 cells. We estimated the quantity of macrophages in transgenic *Tg(mfap4:turquoise)^{x127}* larvae,
122 where macrophages are marked by turquoise fluorescent protein, and found similar numbers of
123 macrophages in larvae that were injected with or immersed in glucose (Figure 1G and 1H). We
124 estimated the quantity of neutrophils in *Tg(lyzC:DsRed)^{nz50}* larvae, where neutrophils are marked by
125 DsRed fluorescent protein, and found reduced numbers of neutrophils in glucose-injected larvae but
126 similar numbers of neutrophils in glucose-immersed larvae (Figure 1I and 1J).

127

128 **Glucose does not affect neutrophil and macrophage recruitment to wounds in zebrafish**
129 **larvae.**

130 Altered innate immune cell recruitment to wounds is a conserved feature of hyperglycaemia in
131 mammals^{4,5,33-35}. To determine if this phenomenon was conserved in zebrafish larvae, we utilised
132 the tail transection wound model which causes reproducible leukocyte recruitment²⁷ (Figure 2A).
133 We first performed this assay using transgenic *Tg(mfap4:turquoise)^{xt27}* larvae to quantify
134 macrophage recruitment to the tail wound (Figure 2B). Surprisingly, we observed no difference in
135 macrophage recruitment between the glucose-injected and control larvae at 6 hours post wounding
136 (hpw) (Figure 2C). We also observed no difference in macrophage recruitment in the glucose
137 immersion model (Figure 2D).

138

139 We then used *Tg(lyzC:DsRed)^{nz50}* larvae to quantify the recruitment of neutrophils to the tail wound
140 (Figure 2E). We observed great variability between experiments with two out of four experiments
141 finding significantly reduced neutrophil recruitment between the control and glucose-injected larvae
142 at 6 hpw and two out of four experiments finding significantly increased neutrophil recruitment
143 (Figure 2F). Consistently, we did not observe any difference in neutrophil recruitment in the
144 glucose immersion model (Figure 2G).

145

146 Together, these results indicate that zebrafish neutrophil and macrophage recruitment is not affected
147 by exogenous glucose supplementation in zebrafish larvae.

148

149 **Glucose impedes haemostasis in zebrafish larvae**

150 Hyperglycaemia perturbs coagulation and platelet activation in mammals, resulting in ineffective
151 haemostasis^{3,8}. To visualise the effects of high glucose on haemostasis in zebrafish larvae, we first
152 used the *Tg(itga2b:gfp)^{la2}* line to visualise thrombocyte plug formation in the severed blood vessel
153^{25,36}. Following tail transection (Figure 3A), glucose-injected *Tg(itga2b:gfp)^{la2}* larvae demonstrated
154 reduced thrombocyte accumulation (Figure 3B-C). This effect was replicated in glucose-immersed
155 embryos compared to control embryos (Figure 3D).

156

157 To determine if the reduction in thrombocyte recruitment was mirrored by perturbed clotting, we
158 conducted tail transections on *Tg(fabp10a:fgb-gfp)^{mi4001}* larvae (Figure 3E), which express
159 fluorescently tagged fibrinogen and allow visualisation of clots ²⁶. Glucose-injected
160 *Tg(fabp10a:fgb-gfp)^{mi4001}* larvae had reduced fibrin accumulation following tail transection
161 compared to PBS-injected larvae (Figure 3F). We did not observe any effect of glucose immersion
162 on the deposition of fluorescently tagged fibrinogen (Figure 3G).

163

164 Together, these results demonstrate that a high glucose environment inhibits the thrombocyte
165 component of haemostasis in zebrafish larvae.

166

167 **High glucose accelerated hyperlipidaemia in zebrafish larvae challenged with a high fat diet**

168 Hyperglycaemia and hyperlipidaemia are intimately associated in mammals ³⁷. To determine if this
169 interaction is conserved in zebrafish larvae, we fed glucose-injected larvae a high fat diet consisting
170 of emulsified chicken egg yolk from 5-7 dpf (Figure 4A). Glucose- and PBS-injected larvae had
171 similar Oil Red O vascular staining prior to the initiation of chicken egg yolk feeding at 5 dpf
172 (Figure 4B-C). Quantification of Oil Red O staining revealed glucose-injected larvae had increased
173 vascular lipid content at one and two days post feeding (Figure 4D-E).

174

175 These results recapitulate the interaction between hyperglycaemia and hyperlipidaemia seen in
176 mammals ¹⁵, demonstrating that zebrafish larvae are a robust model to study this conserved
177 interaction.

178

179 **DISCUSSION**

180 In this study, we explored the effect of high glucose levels on inflammation, thrombosis, and lipid
181 accumulation in the zebrafish embryo model. We found that high glucose reduces haemostasis

182 around wound sites. In addition, we determined that high glucose accelerates lipid accumulation
183 when larvae are challenged with a high fat diet.

184

185 The reduced size of glucose and mannitol immersed larvae compared to control larvae is a major
186 caveat when interpreting datasets generated with the glucose immersion model. We thus preferred
187 the injection method for the chicken egg yolk challenge experiments and for the interpretation of
188 the effects of glucose on larval zebrafish immunity and haemostasis. Despite daily changes of
189 media, we experienced further difficulty rearing zebrafish larvae from 2 to 5 dpf in 5% glucose
190 solutions due to microbial overgrowth which may have slowed larval development by consuming
191 oxygen or damaging larval mucosal surfaces. Although the glucose injection model did not achieve
192 as high a fold change in glucose at 5 dpf, we did not observe developmental delays and
193 haematopoiesis was largely comparable to PBS-injected control larvae.

194

195 Hyperglycaemia in mammals causes vascular dysfunction that restricts the recruitment of a broad
196 range of immune cells to wounds¹⁶. It was therefore surprising that high glucose appeared to have
197 no effect on the recruitment of leukocytes to the tail wound in this study. It is possible that high
198 glucose in zebrafish larvae does not affect abluminal crawling of leukocytes along blood vessels,
199 since wound-responsive leukocytes have been demonstrated to move predominantly through
200 interstitial tissue in zebrafish larvae^{5,38}. Overall our findings suggest exogenous supply of glucose
201 to zebrafish larvae may not be a suitable platform for studying the impact of high glucose on
202 leukocyte biology.

203

204 The relationship between hyperglycaemia and hyperlipidaemia has been previously reported in
205 various mammalian species^{15,32,39}, and a recent study by Wang *et al.* has demonstrated a similar the
206 interaction of a high cholesterol diet with glucose immersion on vascular lipid accumulation in
207 zebrafish after 10 days of feeding³². Our study using chicken egg yolk as a high fat diet challenge

208 demonstrates a dramatically accelerated accumulation of lipid after just one day of feeding. This
209 chicken egg yolk feeding-based model, therefore provides a more rapid model to investigate lipid
210 accumulation.

211

212 In summary, we report glucose-supplemented zebrafish larvae as a tractable platform to investigate
213 the conserved interactions between glucose and haemostasis, and glucose and diet-induced
214 hyperlipidaemia.

215

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220

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226

227 **COMPETING INTERESTS**

228 The authors declare no competing interests.

229

230 **DATA AVAILABILITY STATEMENT**

231 The datasets generated during the current study are available from the corresponding author on
232 reasonable request

233

234 REFERENCES

- 235 1 Salazar, J. J., Ennis, W. J. & Koh, T. J. Diabetes medications: Impact on inflammation and wound
236 healing. *J Diabetes Complications* **30**, 746-752, doi:10.1016/j.jdiacomp.2015.12.017 (2016).
- 237 2 Galkowska, H., Wojewodzka, U. & Olszewski, W. L. Low recruitment of immune cells with increased
238 expression of endothelial adhesion molecules in margins of the chronic diabetic foot ulcers. *Wound*
239 *Repair and Regeneration* **13**, 248-254, doi:10.1111/j.1067-1927.2005.130306.x (2005).
- 240 3 Vazzana, N., Ranalli, P., Cuccurullo, C. & Davì, G. Diabetes mellitus and thrombosis. *Thrombosis*
241 *Research* **129**, 371-377, doi:10.1016/j.thromres.2011.11.052 (2012).
- 242 4 Sawaya, A. P. *et al.* Deregulated immune cell recruitment orchestrated by FOXM1 impairs human
243 diabetic wound healing. *Nature communications* **11**, 1-14, doi:10.1038/s41467-020-18276-0 (2020).
- 244 5 Son, S. M., Kim, I. J. & Kim, Y. K. in *Diabetes* Vol. 49 A147 (2000).
- 245 6 Lucas, T. *et al.* Differential roles of macrophages in diverse phases of skin repair. *Journal of*
246 *immunology (Baltimore, Md. : 1950)* **184**, 3964-3977, doi:10.4049/jimmunol.0903356 (2010).
- 247 7 Miriam, A. M. L. *et al.* Differences in Cellular Infiltrate and Extracellular Matrix of Chronic Diabetic
248 and Venous Ulcers Versus Acute Wounds. *Journal of Investigative Dermatology* **111**, 850,
249 doi:10.1046/j.1523-1747.1998.00381.x (1998).
- 250 8 Rao, A. K. *et al.* Alterations in insulin-signaling and coagulation pathways in platelets during
251 hyperglycemia-hyperinsulinemia in healthy non-diabetic subject. *Thrombosis Research* **134**, 704-
252 710, doi:10.1016/j.thromres.2014.06.029 (2014).
- 253 9 Tsimmerman, G. *et al.* Involvement of microparticles in diabetic vascular complications. *Thromb*
254 *Haemost* **106**, 310-321, doi:10.1160/th10-11-0712 (2011).
- 255 10 Ding, Y. *et al.* Platelet-Rich Fibrin Accelerates Skin Wound Healing in Diabetic Mice. *Annals of Plastic*
256 *Surgery* **79**, e15-e19, doi:10.1097/SAP.0000000000001091 (2017).
- 257 11 Babaei, V. *et al.* Management of chronic diabetic foot ulcers using platelet-rich plasma. *Journal of*
258 *Wound Care* **26**, 784-787, doi:10.12968/jowc.2017.26.12.784 (2017).
- 259 12 Okon, E. B., Chung, A. W. Y., Zhang, H., Laher, I. & van Breemen, C. Hyperglycemia and
260 hyperlipidemia are associated with endothelial dysfunction during the development of type 2
261 diabetes. *Canadian Journal of Physiology and Pharmacology* **85**, 562-567, doi:10.1139/Y07-026
262 (2007).
- 263 13 Andreadou, I. *et al.* Hyperlipidaemia and cardioprotection: Animal models for translational studies.
264 *British journal of pharmacology*, doi:10.1111/bph.14931 (2019).
- 265 14 Crawford-Faucher, A. Preventing or Delaying Type 2 Diabetes Mellitus with Diet and Exercise.
266 *American family physician* **98**, 643-644 (2018).
- 267 15 Pulakazhi Venu, V. K. *et al.* Minimizing Hyperglycemia-Induced Vascular Endothelial Dysfunction by
268 Inhibiting Endothelial Sodium-Glucose Cotransporter 2 and Attenuating Oxidative Stress:
269 Implications for Treating Individuals With Type 2 Diabetes. *Canadian Journal of Diabetes* **43**, 510-
270 514, doi:10.1016/j.jcjd.2019.01.005 (2019).
- 271 16 Williams, S. B. *et al.* Acute Hyperglycemia Attenuates Endothelium-Dependent Vasodilation in
272 Humans In Vivo. *Circulation* **97**, 1695-1701, doi:10.1161/01.CIR.97.17.1695 (1998).
- 273 17 Lamharzi, N. *et al.* Hyperlipidemia in Concert With Hyperglycemia Stimulates the Proliferation of
274 Macrophages in Atherosclerotic Lesions. *Diabetes* **53**, 3217, doi:10.2337/diabetes.53.12.3217
275 (2004).
- 276 18 Oyelaja-Akinsipo, O. B., Dare, E. O. & Katare, D. P. Protective role of diosgenin against
277 hyperglycaemia-mediated cerebral ischemic brain injury in zebrafish model of type II diabetes
278 mellitus. *Heliyon* **6**, doi:10.1016/j.heliyon.2020.e03296 (2020).
- 279 19 Richardson, R. *et al.* Adult Zebrafish as a Model System for Cutaneous Wound-Healing Research.
280 *Journal of Investigative Dermatology* **133**, 1655-1665, doi:<https://doi.org/10.1038/jid.2013.16>
281 (2013).
- 282 20 Martin, P. & Feng, Y. Wound healing in zebrafish. *Nature* **459**, 921-923, doi:10.1038/459921a
283 (2009).
- 284 21 Fang, L., Liu, C. & Miller, Y. I. Zebrafish models of dyslipidemia: relevance to atherosclerosis and
285 angiogenesis. *Translational Research* **163**, 99-108, doi:<https://doi.org/10.1016/j.trsl.2013.09.004>
286 (2014).

- 287 22 Kinkel, M. D. & Prince, V. E. Vol. 31 139-152 (WILEY-VCH Verlag, Weinheim, 2009).
- 288 23 Hall, C., Flores, M. V., Storm, T., Crosier, K. & Crosier, P. The zebrafish lysozyme C promoter drives
289 myeloid-specific expression in transgenic fish. *BMC Dev Biol* **7**, 42 (2007).
- 290 24 Walton, E. M., Cronan, M. R., Beerman, R. W. & Tobin, D. M. The Macrophage-Specific Promoter
291 mfap4 Allows Live, Long-Term Analysis of Macrophage Behavior during Mycobacterial Infection in
292 Zebrafish. *PLoS ONE* **10**, e0138949, doi:10.1371/journal.pone.0138949 (2015).
- 293 25 Lin, H. F. *et al.* Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood* **106**,
294 3803-3810, doi:10.1182/blood-2005-01-0179 (2005).
- 295 26 Vo, A. H., Swaroop, A., Liu, Y., Norris, Z. G. & Shavit, J. A. Loss of fibrinogen in zebrafish results in
296 symptoms consistent with human hypofibrinogenemia. *PLoS ONE* **8**, e74682,
297 doi:10.1371/journal.pone.0074682 (2013).
- 298 27 Cholan, P. M. *et al.* Conserved anti-inflammatory effects and sensing of butyrate in zebrafish. *Gut*
299 *Microbes* **12**, 1-11, doi:10.1080/19490976.2020.1824563 (2020).
- 300 28 Passeri, M. J., Cinaroglu, A., Gao, C. & Sadler, K. C. Hepatic steatosis in response to acute alcohol
301 exposure in zebrafish requires sterol regulatory element binding protein activation. *Hepatology* **49**,
302 443-452, doi:<https://doi.org/10.1002/hep.22667> (2009).
- 303 29 Johansen, M. D. *et al.* Mycobacterium marinum infection drives foam cell differentiation in
304 zebrafish infection models. *Developmental & Comparative Immunology* **88**, 169-172,
305 doi:<https://doi.org/10.1016/j.dci.2018.07.022> (2018).
- 306 30 Matty, M. A., Oehlers, S. H. & Tobin, D. M. Live Imaging of Host-Pathogen Interactions in Zebrafish
307 Larvae. *Methods Mol Biol* **1451**, 207-223, doi:10.1007/978-1-4939-3771-4_14 (2016).
- 308 31 Rocha, F. *et al.* Glucose overload in yolk has little effect on the long-term modulation of
309 carbohydrate metabolic genes in zebrafish (Danio rerio). *The Journal of Experimental*
310 *Biology* **217**, 1139-1149, doi:10.1242/jeb.095463 (2014).
- 311 32 Wang, Z., Mao, Y., Cui, T., Tang, D. & Wang, X. L. Impact of a Combined High Cholesterol Diet and
312 High Glucose Environment on Vasculature. *PLoS ONE* **8**, e81485, doi:10.1371/journal.pone.0081485
313 (2013).
- 314 33 Delamare, M. *et al.* Impaired Leucocyte Functions in Diabetic Patients. *Diabetic Medicine* **14**, 29-34,
315 doi:10.1002/(SICI)1096-9136(199701)14:1<29::AID-DIA300>3.0.CO;2-V (1997).
- 316 34 Wong, S. L. *et al.* Diabetes primes neutrophils to undergo NETosis, which impairs wound healing.
317 *Nature medicine* **21**, 815-819, doi:10.1038/nm.3887 (2015).
- 318 35 Zykova, S. N. *et al.* Altered cytokine and nitric oxide secretion in vitro by macrophages from diabetic
319 type II-like db/db mice. *Diabetes* **49**, 1451, doi:10.2337/diabetes.49.9.1451 (2000).
- 320 36 Huarng, M. C. & Shavit, J. A. Simple and rapid quantification of thrombocytes in zebrafish larvae.
321 *Zebrafish* **12**, 238-242, doi:10.1089/zeb.2014.1079 (2015).
- 322 37 Goldberg, I. J. Diabetic Dyslipidemia: Causes and Consequences. *The Journal of Clinical*
323 *Endocrinology & Metabolism* **86**, 965-971, doi:10.1210/jcem.86.3.7304 (2001).
- 324 38 Barros-Becker, F., Lam, P.-Y., Fisher, R. & Huttenlocher, A. Live imaging reveals distinct modes of
325 neutrophil and macrophage migration within interstitial tissues. *Journal of Cell Science* **130**, 3801,
326 doi:10.1242/jcs.206128 (2017).
- 327 39 Chait, A. & Bornfeldt, K. E. Diabetes and atherosclerosis: is there a role for hyperglycemia? *Journal*
328 *of lipid research* **50 Suppl**, S335-S339, doi:10.1194/jlr.R800059-JLR200 (2009).

329

330 Figure legends

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

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

334 B. Relative concentration of glucose in 5 dpf larvae immersed in 5% solutions of glucose or
335 mannitol from 2 dpf. Statistical testing by ANOVA, each data point is representative of a group of n
336 = 10-30 larvae.

337 C. Total body area calculated from lateral images of 5 dpf glucose-injected larvae. Statistical testing
338 by t-test. Data are representative of 2 biological replicates.

339 D. Total body area calculated from lateral images of 5 dpf larvae immersed in 5% solutions of
340 glucose or mannitol from 2 dpf. Statistical testing by ANOVA. Data are representative of 2
341 biological replicates.

342 E. Eye area calculated from lateral images of 5 dpf glucose-injected larvae. Statistical testing by t-
343 test. Data are representative of 2 biological replicates.

344 F. Eye area calculated from lateral images of 5 dpf larvae immersed in 5% solutions of glucose or
345 mannitol from 2 dpf. Statistical testing by ANOVA. Data are representative of 2 biological
346 replicates.

347 G. Quantification of total macrophage number from lateral images of 5 dpf glucose-injected larvae.
348 Statistical testing by t-test. Data are representative of 2 biological replicates.

349 H. Quantification of total macrophage number from lateral images of 5 dpf larvae immersed in 5%
350 solutions of glucose or mannitol from 2 dpf. Statistical testing by ANOVA. Data are representative
351 of 2 biological replicates.

352 I. Quantification of total neutrophil number from lateral images of glucose-injected 5 dpf larvae.
353 Statistical testing by t-test. Data are representative of 2 biological replicates.

354 J. Quantification of total neutrophil number from lateral images of 5 dpf larvae immersed in 5%
355 solutions of glucose or mannitol from 2 dpf. Statistical testing by ANOVA. Data are representative
356 of 2 biological replicates.

357

358 Figure 2: High glucose does not affect neutrophil and macrophage recruitment to a tail wound.

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

- 360 B. Representative images of macrophage (red) recruitment to a tail wound in glucose-injected
361 larvae.
- 362 C. Quantification of macrophage recruitment following tail transection in the glucose injection
363 model.
- 364 D. Quantification of macrophage recruitment following tail transection in the glucose immersion
365 model.
- 366 E. Representative images of neutrophil (red) recruitment to a tail wound in glucose-injected larvae.
- 367 F. Quantification of neutrophil recruitment following tail transection in the glucose injection model.
- 368 Each paired data point represents the average of an biological replicate with $n > 10$ embryos per
369 condition.
- 370 G. Quantification of neutrophil recruitment following tail transection in the glucose immersion
371 model.
- 372 Scale bars represent 100 μm . Statistical testing by t-test. Data are representative of 3 biological
373 replicates.
- 374
- 375 Figure 3: High glucose reduced thrombocyte and fibrin accumulation at a tail wound.
- 376 A. Schematic of experiment to visualise haemostasis following tail transection.
- 377 B. Representative overlay of thrombocytes (red) at 2.5 hours after tail transection in glucose-
378 injected larvae.
- 379 C. Quantification of thrombocyte plug size following tail transection in the glucose injection model.
- 380 D. Quantification of thrombocyte plug size following tail transection in the glucose immersion
381 model.
- 382 E. Representative images of fibrinogen deposition (red) at 2.5 hours after tail transection in glucose-
383 injected larvae.
- 384 F. Quantification of fibrin clot size following tail transection in the glucose injection model.
- 385 G. Quantification of fibrin clot size following tail transection in the glucose immersion model.

386 Scale bars represent 100 μ m. Statistical testing by t-test. Data are representative of 3 biological
387 replicates.

388

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

390 A. Schematic of the high fat feeding challenge assay to measure lipid accumulation in glucose-
391 injected larvae.

392 B. Bright field images of 6 dpf Oil Red O-stained larvae, demonstrating darker vascular staining in
393 glucose-injected larvae. Box indicates location of inset, arrowheads indicate stained intersegmental
394 vessels in inset, asterisk indicates intestinal lumen which was excluded from analysis.

395 C. Quantification of lipid accumulation in 5 dpf glucose-injected larvae.

396 D. Quantification of lipid accumulation in 6 dpf glucose-injected larvae challenged with a high fat
397 diet from 5 dpf.

398 E. Quantification of lipid accumulation in 7 dpf glucose-injected larvae challenged with a high fat
399 diet from 5 dpf.

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







