Embryonic nutritional hyperglycemia decreases cell proliferation in the zebrafish retina

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Short Report

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Abstract

Diabetic retinopathy (DR) is one of the leading causes of blindness in the world. While there is a major focus on the study of juvenile/adult DR, the effects of hyperglycemia during early retinal development are less well studied. Recent works in embryonic zebrafish models of nutritional hyperglycemia revealed that hyperglycemia leads to decreased cell numbers of mature retinal cell types, which has been related to a modest increase in apoptotic cell death and altered cell differentiation (Singh et al. 2019; Titialii-Torres and Morris 2022). However, how embryonic hyperglycemia impacts cell proliferation in developing retinas remains still unknown. Here, we exposed zebrafish embryos to 50 mM glucose from 10 hours postfertilization (hpf) to 5 days postfertilization (dpf). First, we confirmed that hyperglycemia increases apoptotic death and decreases the rod and Müller glia population in the retina of 5 dpf zebrafish. Interestingly, the increase in cell death was mainly observed in the ciliary marginal zone (CMZ), where most of the proliferating cells are located. To analyze the impact of hyperglycemia in cell proliferation, mitotic activity was first quantified using pH3 immunolabeling, which revealed a significant decrease in mitotic cells in the retina (mainly in the CMZ) at 5 dpf. A significant decrease in cell proliferation in the outer nuclear and ganglion cell layers of the central retina in hyperglycemic animals was also detected using the proliferation marker PCNA. Overall, our results show that nutritional hyperglycemia decreases cellular proliferation in the developing retina, which could contribute significantly to the decline in the number of mature retinal cells.

Introduction

One of the most common neurological complications of diabetes is diabetic retinopathy (DR), which is one of the leading causes of vision loss in developed countries and the principal cause of blindness in working-age adults worldwide (Leasher et al. 2016; Ogurtsova et al. 2017; Pan et al., 2021). Moreover, DR is considered as an independent indicator of other diabetic-related complications like diabetic nephropathy, cardiovascular disease or stroke, and is even related with a higher risk of morbidity and mortality (Simó et al. 2018). Although DR was initially considered a vascular disease, more recent research has shown that it also is a neurodegenerative disease affecting the neural retina and that cellular changes in the retina precede the clinical features (Cai and McGinnis 2016). DR research using animal models of diabetes/hyperglycemia mainly involves the study of juvenile/adult DR (see Cai and McGinnis 2016), which includes the use of juvenile/adult zebrafish models of DR (Ali et al. 2020; Wang et al. 2020; Wiggenhauser et al. 2020; Schmitner et al. 2021). However, the effects of hyperglycemia during early retinal development have been less studied. Hyperglycemia during pregnancy affects the embryo and can have significant effects in a variety of organs in the offspring (Gilbert et al. 2013; Scott-Drechsel et al. 2013; Kua et al. 2019). For example, the offspring of diabetic mothers presents macular defects (Tariq et al. 2010). Genetic and nutritional zebrafish models of diabetes are emerging as tools of interest to understand the cellular/molecular consequences of hyperglycemia in the developing eye (Jung et al. 2016; Singh et al. 2019; Lee and Yang 2021; Titialii-Torres and Morris 2022). Interestingly, two of these studies developed embryonic models of nutritional hyperglycemia in zebrafish embryos to analyze the

effects of high glucose levels in early retinal development (Singh et al. 2019; Titialii-Torres and Morris 2022). Singh et al. (2019) exposed zebrafish embryos from 3 hours postfertilization (hpf) to 5 days postfertilization (dpf) to 4–5% D-glucose (222 mM to 277 mM) in fish water, which led to changes in the thickness of several retinal layers and a decrease in BrdU incorporation in the retina and in the number of ganglion cells and Müller glia. More recently, Titialii-Torres and Morris (2022) exposed zebrafish embryos to 50 mM D-glucose from 10 hpf to 5 dpf. Exposure to 50 mM glucose in fish water led to a reduction of the number of cone and rod photoreceptors and of horizontal cells (Titialli-Torres and Morris 2022). The decrease in mature cell types in the 5 dpf hyperglycemic retina could be related to a modest increase in apoptotic cell death (as shown by TUNEL labeling) and to altered cell differentiation (as shown by changes in EdU incorporation in different layers of the central retina) (Titialli-Torres and Morris 2022). However, despite the relevance of cell proliferation at early developmental stages for proper growth and development of the retina, there is a lack of studies on the effects of embryonic hyperglycemia on mitotic activity and cell proliferation of the early zebrafish retina. These analyses are of crucial importance specially when the first days of development coincide with the peak in the rate of retinal cell proliferation in the entire zebrafish lifespan (Hernández-Núñez et al. 2021b).

Here, the same zebrafish model of embryonic nutritional hyperglycemia of Titialii-Torres and Morris (2022) was replicated to analyze the effects of exposure to high levels of glucose in early retinal cell proliferation. First, our results confirmed the increase in apoptotic cell death, and the decrease in rod photoreceptors and Müller glia in hyperglycemic retinas (Singh et al. 2019; Titialii-Torres and Morris 2022). But, most importantly, our data revealed that nutritional hyperglycemia decreases mitotic activity and cell proliferation in the zebrafish retina. Our data suggests that impaired cell proliferation of neural progenitor cells could be an important factor contributing to the decreased numbers of mature retinal cells in the developing hyperglycemic retina.

Material And Methods

Animals

10 hpf wild type zebrafish (*Danio rerio*) specimens were used in this study. Adult zebrafish used for embryo generation were kept in aquaria under standard conditions of temperature (28 ºC), light-dark cycles (14 h of light and 10 h of darkness), and pH (7.0). Adult fish of both sexes were crossed to generate embryos, which were raised using standard conditions until being used for experimental procedures (at 10 hpf). The study was conducted according to the regulations and laws established by the European Union (2010/63/UE) and by the Spanish Royal Decree 1386/2018 for the care and handling of animals in research and was approved by the Bioethics Committee of the University of Santiago de Compostela and the Xunta de Galicia (project reference MR 110250).

Generation of embryonic nutritional hyperglycemia

10 hpf embryos were placed in groups of 5 into 24 well-plates containing 2 mL per well of reverse osmosis purified water supplemented (hyperglycemic fish) or not (control embryos) with 50 mM D-

glucose (Sigma, St Louis, MO, USA). The water (with or without D-glucose) was replaced once a day for 5 days. At 5 dpf, larvae were anesthetized with 0.02% of ethyl 3-aminobenzoate methanesulfonate salt (MS-222, Tricaine; Sigma) and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate-buffered saline pH 7.4 (PBS) for 2 hours at 4 ºC. After rinsing in PBS, the larvae were cryoprotected with 30% sucrose in PBS, embedded in Neg-50™ (Thermo Scientific, Kalamazoo, MI, USA), and frozen with liquid nitrogen-cooled isopentane. Transverse sections of the head containing the eyes (18 µm thickness) were obtained on a cryostat and mounted on Superfrost Plus slides (Menzel-Glasser, Madison, WI, USA).

TUNEL labeling

The TUNEL staining was performed according to the manufacturer's protocol with minor modifications (In situ Cell Death Detection Kit, TMR red; catalogue number 12156792910; Roche, Mannheim, Germany). Briefly, the sections were incubated in methanol for 15 minutes at -20°C to permeabilize the lipid membranes, followed by brief washes in PBS and another incubation in 0.01 M citrate buffer pH 6.0 for 30 min at 90 ºC. After several washes in PBS, the sections were incubated in the TUNEL reaction mix, containing the Labelling Solution (TMR red labelled nucleotides) and the Enzyme Solution (terminal deoxynucleotidyl transferase), for 90 minutes at room temperature (RT). Slides were washed in PBS and distilled water, allowed to dry for 45 min at 37 ºC, and mounted with MOWIOL® 4–88 (Calbiochem, Darmstadt, Germany). Negative controls were obtained by incubating slides only with the Labelling solution (without terminal deoxynucleotidyl transferase).

Immunofluorescence

Sections were first treated with 0.01 M citrate buffer pH 6.0 for 30 min at 90 ºC for heat-induced epitope retrieval, allowed to cool for 20 min at RT, and rinsed in 0.05 M Tris-buffered saline (TBS) pH 7.4 for 5 min. Then, the sections were incubated overnight at RT with the following combinations of primary antibodies: 1) a mouse monoclonal anti-PCNA antibody (1:500; Sigma; catalogue number P8825; RRID: AB_477413) and a rabbit polyclonal anti-pH3 antibody (1:300; Millipore; Billerica, MA, USA; catalogue number 06-570; RRID: AB_310177); 2) rabbit polyclonal anti-bovine rod rhodopsin antibody [CERN-922; 1:500; generated by W. J. Degrip (Radboudumc, The Netherlands)] and a mouse monoclonal anti-glutamine synthetase (GS) (1:500; Millipore; catalogue number MAB302; RRID: AB_2314617). Sections were then rinsed 3 times in TBS for 10 min each and incubated for 1 hour at RT with a combination of fluorescent dye-labelled secondary antibodies: a Cy3-conjugated goat anti-rabbit (1:200; Invitrogen, Waltham, MA, USA; catalogue number A10520; RRID: AB_2534029) and a FITC-conjugated goat anti-mouse (1:200; Invitrogen; catalogue number F2761; RRID: AB_1500661). All antibody dilutions were made in TBS containing 15% normal goat serum (Millipore), 0.2% Triton X-100 (Sigma) and 2% BSA (Sigma). Finally, sections were rinsed 3 times in TBS for 10 min each and in distilled water for 30 min, allowed to dry for 45 min at 37 ºC, and mounted in MOWIOL® 4–88 (Calbiochem).

Specificity of antibodies

Anti-PCNA antibodies (including the one used in this study) have been traditionally used to label proliferating cells in the retina of different fish species (including zebrafish): *Oryzias latipes* (Negishi et al.

1990); *Haplochromis burtoni* (Mack and Fernald 1995, 1997); *Onchorynchus mykiss* (Julian et al. 1998); *Tinca tinca* (Velasco et al. 2001; Cid et al. 2002; Jimeno et al. 2003); *Carassius auratus* (Negishi et al. 1990; Cid et al. 2002); *Salmo trutta fario* (Candal et al. 2005); *Astyanax mexicanus* (Alunni et al. 2007); *D. rerio* (Cid et al. 2002; Amini et al. 2019; Hernández-Núñez et al. 2021b); *Scyliorhinus canicula* (Ferreiro-Galve et al. 2010a, 2010b, 2012; Sánchez-Farías and Candal 2015, 2016; Hernández-Núñez et al. 2021a); and *Petromyzon marinus* (Villar-Cheda et al. 2008). Anti-pH3 antibodies (including the one used in this study) have been also commonly used to label mitotic cells in the retina of various fish species (including zebrafish): *C. auratus* (Otteson et al. 2001); *S. canicula* (Ferreiro-Galve et al. 2010a; Bejarano-Escobar et al. 2012; Hernández-Núñez et al. 2021a); and *D. rerio* (Jensen et al. 2001; Godinho et al. 2007; Weber et al. 2014; Hernández-Núñez et al. 2021b). The location of labeled cells with both antibodies (see results) in stereotypical locations containing proliferating cells in the highly organized retina also ensures the specificity of labeling. The anti-bovine rod rhodopsin antibody (CERN-922) has been previously shown to label specifically rod photoreceptors in the retina of lampreys (Meléndez-Ferro et al. 2002; Villar-Cheda et al. 2008) and teleosts (trout: Candal et al. 2005; tench: Bejarano-Escolar et al. 2009). The morphology and location of labelled rods in the zebrafish retina also ensures the specificity of labeling. Anti-GS antibodies have been widely used as specific markers of Müller glia cells in the retina of fish, including teleosts as *H. burtoni* (Mack et al. 1998) and *D. rerio* (Peterson et al. 2001; Thummel et al. 2008); and in the elasmobranch fish *S. canicula* (Sánchez-Farías and Candal 2016; Hernández-Núñez et al. 2021a). As with other antibodies, the location and morphology of labelled cells in the highly organized zebrafish retina ensures the specificity of labeling.

Image acquisition

Images of fluorescent labelled sections were taken with Leica TCS-SP2 (CERN-922) or Leica Stellaris 8 (pH3, PCNA, TUNEL and GS) confocal microscopes with blue and green excitation lasers. 20x and 40x objectives were used for imaging. Confocal optical sections were taken at steps of 1 µm along the z-axis. Collapsed images of the whole retinal sections (18 µm) were obtained with LITE or LAS X software (Leica, Wetzlar, Germany). For figure preparation, and always after cell quantifications, contrast and brightness of the images were minimally adjusted using Adobe Photoshop CS4 (Adobe, San Jose, CA, USA).

Cell quantification and statistical analyses

We quantified the number of apoptotic (TUNEL+) and mitotic cells (pH3+) in the whole retina (CMZ and central retina), and the number of cells progressing through the cell cycle (PCNA+) in the central retina. The numbers of TUNEL+, pH3 + and PCNA + cells were manually counted under an Olympus fluorescence microscope in 1 out of each 2 consecutive retinal sections (thickness of 18 µm). The boundary between the CMZ and the central retina was established based on the expression pattern of PCNA, which shows intense CMZ labeling, and the different morphology of the CMZ and the central retina (which shows a characteristic layered structure). With the number of cells for each quantified section we calculated the mean number of cells per section for each retina (each dot in the graph represents 1 retina from 1 animal). We also quantified the differential distribution of PCNA + cells in the different cell layers of the

central retina: the ganglion cell layer (GCL), the inner nuclear layer (INL), and the outer nuclear layer (ONL).

The number of GS-ir or CERN-922-ir positive pixels was quantified in confocal photomicrographs of entire retinal sections using the Feature J plugin in the Fiji software (Schindelin et al. 2012) and as previously described for quantification of immunoreactive (ir) profiles in lamprey spinal cord sections (Fernández-López et al. 2014; Romaus-Sanjurjo et al. 2018). Briefly, the retinal images were always taken with the same confocal microscope and acquisition software parameters for control and hyperglycemic retinal sections. The number of positive pixels was quantified in 1 out of each 2 consecutive retinal sections. Then, the mean number of positive pixels per section was obtained for each retina (each dot in the graphs represents 1 retina from 1 animal). A threshold was established to have the most representative images when converting them to binary B&W images for pixel quantification. The same threshold was used for all the photomicrographs with the same labeling (GS or CERN-922).

Statistical analyses were performed with Prism 9 (GraphPad software, La Jolla, CA, USA). Normality of the data was determined with the D'Agostino-Pearson test. To determine significant differences ($p < 0.05$) between control and hyperglycemic animals, a Mann-Whitney test was used for non-normally distributed data and a Student's (unpaired) t-test for normally distributed data. Each dot in the graphs represents 1 retina from 1 animal and all analyses were carried out in at least 2 different batches of animals.

Results And Discussion

Embryonic nutritional hyperglycemia alters retinal development in zebrafish

To model embryonic nutritional hyperglycemia in developing zebrafish we exposed zebrafish embryos to 50 mM D-glucose from 10 hpf (prior to the optic vesicle evagination) to 5 dpf. This covers the entire early retinal development, since a highly organized central retina can be observed from 2.5 dpf (Malicki et al 1996) and the retina is functional at about 3 dpf (Easter and Nicola 1996). As shown by Titialii-Torres and Morris (2022), this exposure to glucose causes a significant increase in whole-body glucose levels in 5 dpf zebrafish larvae. To confirm the effects of embryonic hyperglycemia in 2 of the mature retinal cell types (rods and Müller glia) we analyzed changes in the expression of rod rhodopsin (CERN-922 antibody) and GS (which specifically labels Müller glia cells of the central retina).

We observed a significant decrease in rod photoreceptors (Fig. 1a-c) as shown by the 26% decrease in the number of CERN-922 positive pixels in the retina of hyperglycemic animals. This coincides well with the 34% decrease in the number of rod photoreceptors observed by Titialii-Torres and Morris (2022) with the same 50 mM D-glucose exposure and using a XOPS:GFP transgenic zebrafish line that labels rods. Interestingly, numbers of rod photoreceptors are not recovered at 12 dpf even after returning larvae to normoglycemic conditions (Titialii-Torres and Morris 2022).

We also observed a significant decrease in Müller glia cells (Fig. 1d-f) as shown by the 40% decrease in the number of GS positive pixels in the retina of hyperglycemic animals. This coincides well with the 37% loss of GFAP:GFP + Müller glia cells observed by Singh et al. (2019) after exposure to 4% (222 mM) D-glucose from 24 to 48 hpf. Interestingly, in the study by Singh et al. (2019) numbers of Müller glia cells did not recover to control levels after a 24 hour recovery period without glucose, which is similar to the lack of photoreceptor recovery (see above). In contrast, Titialii-Torres and Morris (2022) did not observe a significant change in GFAP:GFP + Müller glia cell numbers in hyperglycemic animals at 5 dpf after exposure to 50mM D-glucose. Titialii-Torres and Morris (2022) proposed that the discrepancy between their data and that of Singh et al. (2019) using the same GFAP:GFP marker could be related to the use of different concentrations of glucose. Interestingly, exposure of adult zebrafish to 2% glucose (111 mM) did not significantly change GFAP expression levels in the retina (Tanvir et al., 2018). However, our data using GS as a marker shows that 50 mM glucose exposure also affects Müller glia. This indicates that GS might be a better marker to reveal the effects of hyperglycemia in zebrafish Müller glia as compared to GFAP, which did not reveal these changes with the same glucose concentration (Titialli-Torres and Morris 2022).

Our analysis of TUNEL labeling revealed a significant increase in the number of apoptotic cells in the whole retina of hyperglycemic animals (Fig. 1g-k). When looking separately at the CMZ and central retina we observed a significant increase in TUNEL positive cells in the CMZ (Fig. 1j) and no significant changes in cell death in the central retina (Fig. 1k). Titialii-Torres and Morris (2022) observed a modest increase in cell death in the 50 mM D-glucose treated retina, which was primarily noted in the GCL and INL at 4dpf. As indicated by Titialii-Torres and Morris (2022) with qualitative observations, with our quantitative date we detected more TUNEL positive cells in the central retina than in the CMZ. However, our data looking separately at the CMZ and the central retina indicates that the increase in cell death observed in hyperglycemic animals occurs mainly in the CMZ.

Our results, and those of others (Singh et al. 2019; Titialii-Torres and Morris 2022), confirm that embryonic hyperglycemia causes a reduction of mature cell types in the central retina, which leads to morphological/structural changes in the retina (Singh et al. 2019; our own observations). Titialii-Torres and Morris (2022) proposed that the decrease in mature cell numbers could be attributed to the modest increase in cell death in the central retina and to changes in cell differentiation (as shown by changes in EdU labeling in the different layers of the central retina). But our TUNEL labeling analyses show that cell death is mainly increased in the CMZ, which is where most of the retinal progenitor cells are located and where most of the mitotic activity occurs (Hernández-Núñez et al. 2021b). This, together with the lack of regeneration of mature cell types after a period in normoglycemic conditions (Singh et al. 2019; Titialii-Torres and Morris 2022), leads us to hypothesize that cell proliferation might be impaired in progenitor cells during embryonic nutritional hyperglycemia.

Embryonic Nutritional Hyperglycemia Decreases Cell Proliferation In The Zebrafish Retina

To analyze the impact of embryonic hyperglycemia in cell proliferation, we first evaluated mitotic activity using pH3 immunolabeling. Embryonic hyperglycemia caused a significant decrease in the number of mitotic cells in the whole retina of 5 dpf zebrafish (Fig. 2a-c). This loss of mitotic activity was predominantly observed in the CMZ (Fig. 2d) as shown by a separate quantification of pH3 + cells in the CMZ (Fig. 2d) and central retina (Fig. 2e). The use of PCNA labeling (which detects different stages of the cell cycle and allows to detect a higher number of proliferating cells in the central retina; Hernández-Núñez et al. 2021b), allowed us to further explore proliferative activity in the central retina. Quantification of PCNA + cells in the central retina revealed a significant decrease in proliferating cells in 5 dpf hyperglycemic animals (Fig. 2f) Separate quantifications of the three cell layers of the central retina (Fig. 2g-i) revealed that the decrease in PCNA labelling predominantly occurs in the outer nuclear (Fig. 2g) and ganglion cell layers (Fig. 2i) of the central retina. Singh et al. (2019) also reported a decrease in cell proliferation in 5 dpf animals after exposure to 4 or 5% D-glucose from 3 hpf. However, they analyzed this by using BrdU labeling, which would also be retained by differentiated cells after exiting the cell cycle. Similar observations have been reported in adult mutant models of DR in zebrafish using EdU labeling (Schmitner et al. 2021), which, as BrdU, would be also retained by differentiated/mature cells. Thus, our results show for the first time by using specific markers of mitotic activity and cell proliferation that nutritional hyperglycemia decreases cell proliferation in progenitor cells of the developing zebrafish retina, which could be an important contributor to the decrease in the number of mature retinal cell types. Future work should try to determine the molecular and cellular mechanisms by which nutritional hyperglycemia alters cell proliferation in progenitor cells of the embryonic zebrafish retina. Previous work revealed that embryonic nutritional hyperglycemia causes an increase in the generation of reactive oxygen species (Titialii-Torres and Morris 2022) and an increase in macrophage numbers (Singh et al. 2019) in the zebrafish retina but a link between these factors and inhibition of cell proliferation remains to be established. Also, and due to the importance of Müller glia in the general homeostasis of the retina (Bringmann et al. 2006), it could be of interest to study the relationship between the loss of Müller glia cells (present results; Singh et al. 2019) and the decrease in cell proliferation. In addition, exposure of zebrafish embryos to 130 mM glucose from 3 to 6 dpf leads to pathological changes in the hyaloid-retinal vessels (Jung et al. 2016). Thus, it would be of interest to study the possible relationship between vessel pathology and decreased cell proliferation in the retina of developing hyperglycemic animals. The generation and deep characterization of this zebrafish model of embryonic nutritional hyperglycemia will also allow for the screen of candidate drugs to revert the decrease in mature retinal cell types by promoting cell survival, and/or proliferation and differentiation from progenitor cells.

Declarations

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Author contributions

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Competing interests

The authors declare no financial or non-financial competing interests.

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Figures

Figure 1

Embryonic nutritional hyperglycemia alters retinal development. Exposure to 50 mM D-glucose causes a decrease in rod photoreceptors (CERN-922 antibody) at 5 dpf (**a-c**). Exposure to 50 mM D-glucose causes a decrease in Müller glia (anti-GS antibody) at 5 dpf (d-f). Exposure to 50 mM D-glucose causes an increase in cell death (TUNEL labeling) at 5 dpf (**g-i**). The increase in cell death is statistically significant the CMZ (**j**) but not in the central retina (**k**). Dashed lines mark the limit between the CMZs and the central retina. Arrowheads indicate TUNEL+ cells. Scale bars: 50 µm. ns: non-significant; *: $p < 0.05$; **: $p < 0.01$.

Figure 2

Embryonic nutritional hyperglycemia decreases cell proliferation in the 5 dpf retina. **a.** pH3 and PCNA immunolabeling in a control retina. **b.** pH3 and PCNA immunolabeling in a hyperglycemic retina. Nutritional hyperglycemia causes a significant decrease in mitotic activity (pH3) in the whole retina (**c**) and in the CMZ (**d**) but not in the central retina (**e**). Nutritional hyperglycemia causes a significant decrease in cell proliferation (PCNA) in the central retina (**f**). Nutritional hyperglycemia causes a significant decrease in cell proliferation (PCNA) in the ONL (**g**) and GCL (**i**) but not in the INL (**h**). Dashed lines mark the limit between the CMZs and the central retina. Arrowheads indicate pH3+ cells of the CMZ. Arrows indicate PCNA+ cells of the central retina. Scale bars: 50 µm. ns: non-significant; *: $p < 0.05$; **: $p < 0.01$.