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High-glucose/high-cholesterol diet in zebrafish evokes diabetic and affective pathogenesis: The role of peripheral and central inflammation, microglia and apoptosis

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ABSTRACT

Neuroinflammation and metabolic deficits contribute to the etiology of human affective disorders, such as anxiety and depression. The zebrafish (*Danio rerio*) has recently emerged as a powerful new model organism in CNS disease modeling. Here, we exposed zebrafish to 2% glucose and 10% cholesterol for 19 days to experimentally induce type 2 diabetes (DM) and to assess stress responses, microglia, inflammation and apoptosis. We analyzed zebrafish anxiety-like behavior in the novel tank and light-dark box (Days 15–16) tests, as well as examined their biochemical and genomic biomarkers (Day 19). Confirming DM-like state in zebrafish, we found higher whole-body glucose, triglyceride, total cholesterol, low-density lipoprotein levels and glucagon mRNA expression, and lower high-density lipoprotein levels. DM zebrafish also showed anxiety-like behavior, elevated whole-body cortisol and cytokines IFN-γ and IL-4, as well as higher brain mRNA expression of the glucocorticoid receptor, CD11b (a microglial biomarker), pro-inflammatory cytokines IL-6 and TNF-α (but not IL-1β or anti-inflammatory cytokines IL-4 and IL-10), GFAP (an astrocytal biomarker), neurotrophin BDNF, its receptors p75 and TrkB, as well as apoptotic *Bax* and *Caspase-3* (but not *BCl-2*) genes. Collectively, this supports the overlapping nature of DM-related affective pathogenesis and emphasizes the role of peripheral and central inflammation and apoptosis in DM-related affective and neuroendocrine deficits in zebrafish.

1. Introduction

Diabetes mellitus (DM) and other metabolic disorders are life-threatening pathologies that are increasingly prevalent globally (Whiting et al., 2011; Wild et al., 2004). The type 2 DM (T2DM) involves aberrant glucose metabolism (Capiotti et al., 2014b; Intine et al., 2013) that causes retinal and vascular deficits (Gleeson et al., 2007; Zemin et al., 2013) and impaired neurogenesis (Dorsemans et al., 2018). DM is also associated with affective disorders, including anxiety and depression (Collins et al., 2010; Lustman, 1988; Simon et al., 2005; Smith et al., 2013). For example, 10–15% of individuals with DM suffer from depression (Kampling et al., 2017), which is twice more prevalent in DM patients (Mezuk et al., 2008; Stuart and Baune, 2012). They also have higher risks of developing anxiety disorders (Goldney et al., 2004; Nouwen et al., 2010), collectively supporting a complex pathogenetic interplay between DM and affective disorders. However, their potentially overlapping mechanisms remain poorly understood, necessitating further translational research and new integrative disease models.

Animal models are an indispensable tool to study both metabolic (Al-Awar et al., 2016; Buettner et al., 2012) and affective disorders (Ashkenazyfrolinger et al., 2015; Bilu et al., 2016; Fonseka et al., 2015; Ransome et al., 2012; Song et al., 2018; Ziv et al., 2012). The zebrafish

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(*Danio rerio*) has recently emerged as a powerful novel model organism in biomedicine (Alestrom et al., 2006; Brittijn et al., 2009; Tavares and Santos, 2013) and translational neuroscience (Kalueff et al., 2014a; Mezzomo et al., 2017; Stewart et al., 2014). Zebrafish display robust affective phenotypes, and are highly sensitive to acute or chronic stress (Cachat et al., 2011, Egan et al., 2009, Pavlidis et al., 2015, Grzelak et al., 2017), as well as to its genetic and pharmacological modulation (Bailey et al., 2015; Barros et al., 2010; Goldsmith, 2004). Like human hypothalamic-pituitary-adrenal (HPA) axis, the zebrafish hypothalamic-pituitary-interrenal (HPI) axis utilizes cortisol as the main stress hormone (Griffiths et al., 2012; Khan et al., 2017). Due to high genetic and physiological homology to humans, zebrafish have also been used to study DM (Jrgens et al., 2012; Maddison and Chen, 2017), including its role in CNS mechanisms (Dorsemans et al., 2017a; Dorsemans et al., 2017b; Lakstygal et al., 2018; Meng et al., 2017). For example, like high-caloric diet (Stankiewicz et al., 2017), experimental hyperglycemia induces anxiety-like behaviors in zebrafish, suggesting these fish as a useful model for targeting stress-DM interplay (Capiotti et al., 2014a; Dorsemans et al., 2018; Yanyi et al., 2017).

Mounting evidence implicates neuroimmune mechanisms and neuroendocrine deficits in both DM and affective disorders (Micale and Drago, 2018; Stuart and Baune, 2012). Because hyperglycemia and hyperlipidemia frequently occur in T2DM, glucose and cholesterol-rich diet has been used for DM modeling in both rodents (Kaiser et al., 2012; Winzell and Ahrén, 2004) and zebrafish (Intine et al., 2013). Capitalizing on these established models, here we examine the impact of experimentally-induced T2DM-like states on zebrafish behavioral and endocrine stress-related phenotypes, with a specific focus on potential roles for microglia, neuroinflammation and apoptosis in this pathogenesis.

2. Materials and methods

2.1. Animal husbandry, experimental design and sample collection

Adult shot-fin wild-type zebrafish (~1:1 male-to-female ratio) were purchased from a local commercial supplier (Yayi Aquarium Shop, Zhanjiang, China) and housed in the Benchtop Aquatic System (Jinshui Marine Biological Equipment Co., Qingdao, China) at 14 h light:10 h dark cycle, 28.5 ± 1 °C, for at least 14 days prior to the study. To induce experimental T2DM, the animals were housed in 6-L tanks in 2% glucose (Sinopharm Chemical Reagent Beijing Co., Beijing, China) (Dorsemans et al., 2018) and fed cholesterol-containing diet (Yina et al., 2013) for 19 days (Fig. 1). Briefly, the control group was fed twice a day (at 12:00 AM and 6:00 PM) with the small-fish special food (YEE, Weifang, China), and the DM group received the same food containing 10% cholesterol (Aladdin, Shanghai, China). The water in holding tanks was changed daily for 19 days, after which zebrafish were sacrificed in iced water, followed by decapitation and brain extraction. Because our DM model involved a combined action of high glucose + high-fat diet, assessing these factors individually in zebrafish was beyond the scope of this study. The brain and body samples were them immediately placed in liquid nitrogen and transferred to a − 80 °C freezer for further analyses. The length and weight of each zebrafish body were also recorded during sampling, to calculate the body-mass index (BMI) as the body weight (g) divided by the square of the body length (cm).

2.2. Behavioral testing

Behavioral testing was performed on Day 15 in the 5-min novel tank test (NTT) and on Day 16 in the 5-min light-dark box (LDB), chosen here as the two most sensitive and commonly used paradigms to assess zebrafish behavior (Kalueff and Cachat, 2011a,b; Kalueff et al., 2014b; Kalueff et al., 2012). The NTT was a Plexiglas 1.5-L trapezoidal tank (15 height × 28 top/23 bottom × 7 width cm) virtually divided into top and bottom halves, assessing the number of top transitions, time spent in top (s) and the latency to enter the top (s), as described earlier (Egan et al., 2009). The LDB was a plastic 15-L box consisting of two chambers (dark and light, each 20 height × 16 length × 24 width cm) (Song et al., 2018), to assess the number of light transitions, time spent in light (s) and the latency to enter the light (s) (Gould, 2011; Stephenson et al., 2011; Stewart et al., 2011).

2.3. Whole-body biochemical analyses

Following decapitation and brain extraction, headless body samples were thawed, homogenized with 0.9% normal saline (1:9 weight/weight) and centrifuged at 4 °C, 2500 rpm for 20 min, collecting supernatant for biochemical analyses. The protein quantitative analysis was performed using the BCA assay (Takara Bio Inc., Shiga, Japan). The levels of glucose, total glyceride, total cholesterol, high- (HDL) and low-density (LDL) lipoproteins were assayed in these whole-body samples using biochemical kits (Jiancheng Company, Nanjing, China) following the manufacturer instructions. The whole-body samples were chosen for this study because of technical difficulties with collecting zebrafish blood (due to small size of these animals) in vivo. The levels of whole-body cortisol, an anti-inflammatory cytokine interleukin (IL) IL-4 and a pro-inflammatory interferon-gamma (IFN-γ) were assayed using the ELISA kits (ZIKE Biotech, Shenzhen, China), following the manufacturer instructions. All data were standardized by the total protein concentrations. Circulating IL-4 and IFN-γ were used here as common biomarkers of peripheral pro-/anti-infmammation, and cortisol was analyzed as a key endocrine biomarker of stress.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression of DM-related genes (glucagon, insulin and its receptor *Insra*) was assayed in the muscle tissue collected from the whole-body samples, similar to (Cadoudal et al., 2008; Das et al., 2016; Zemin et al., 2013). The muscle tissue was chosen here for analyses due to its prominent role in body's glucose metabolism (Cadoudal et al., 2008; Das et al., 2016; Zemin et al., 2013). The expression of genes encoding a key neurotrophin, the brain-derived neurotrophic factor (BDNF) and its two receptors (P75, TrkB), an astrocyte biomarker glial fibrillary acidic protein (GFAP), a microglia biomarker CD11b, the glucocorticoid receptor (GR), selected pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-4, IL-10), IL-6 receptor (IL-6R), as well as major apoptotic genes *Caspase-3*, *Bcl-2* and *Bax*, was assessed in whole-brain samples collected as described above. The whole-brain samples were utilized here for gene expression analyses due to our study's focus on modeling affective pathogenesis and DM-related neurobehavioral deficits in zebrafish.

Trisol was used to lysate the homogenized muscle or brain samples, and the mRNA isolation steps were performed according to the

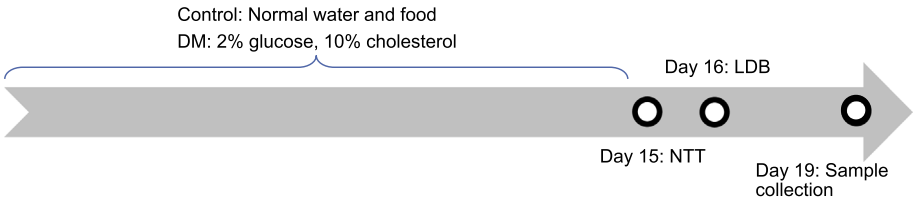

Fig. 1. A brief summary of the study experiment design. NTT – the novel tank test, LDB – the light-dark box test, DM – diabetic experimental group.

Table 1
Primers used in the present study (Sangon Biotech, Shanghai, China), F - forward, R - reverse.

Genes	Sequences
Glucagon F	AAGCGAGGAGACGATCCAAA
Glucagon R	TCCAACACACACCAGCAAATG
Insulin F	GAGCCCCTTCTGGGTTTCC
Insulin R	AAGTCAGCCACCTCAGTTTCCT
Insr a F	GGAGCCCCACTCGTCTAACAAA
Insr a R	CGCCGTTGTGAATGACGTATTC
BDNF F	AACTCCAAAGGATCCGCTCA
BDNF R	GCAGCTCTCATGCAACTGAA
TrkB F	CCACCACTGGAGGACAGAGTTG
TrkB R	CCGAGGATGATGGCGTGTTGT
P75 F	TCTGTCAAGATTTCGATGCTCCT
P75 R	GCTCTCCGTAGGATTGTCCG
GFAP F	AATGTCAAACTGGCCCTGGAT
GFAP R	CTCTCCGTCACGGGTCTCAA
CD11b F	TCCTCGGATTCCAGAAACAC
CD11b R	AGCAGCACAAGTCCTCCAAT
GR F	ACAGCTTCTTCCAGCCTCAG
GR R	CCGGTGTTCTCCTGTTTGAT
IL-1β F	TTCCCCAAGTGCTGCTTATT
IL-1β R	AAGTTAAAACCGCTGTGGTCA
IL-4 F	GCAGGAATGGCTTTGAAGGG
IL-4 R	GCAGTTTCCAGTCCCGGTAT
IL-6 F	TCAACTTCTCCAGCGTGATG
IL-6 R	TCTTTCCCTCTTTTCCTCCTG
IL-6R F	GCCAACTGCAACATACCAAA
IL-6R R	ACTGACAGCACGCAAAACTC
IL-10 F	CTCTGCTCACGCTTCT
IL-10 R	TAGGGACTGTTTATGTTATG
TNF-α F	GGGCAATCAACAAGATGGAAG
TNF-α R	GCAGCTGATGTGCAAAGACAC
Caspase 3 F	CCAGGGTCAACCATAAAGTAGC
Caspase 3 R	TCTTTGGTGAGCATTGAGACGA
Bcl-2 F	TGGATGACTGACTACCTGAAC
Bcl-2 R	GTATGAAAACGGGTGGAAC
Bax F	GTGTATGAGCGTGTTCGTC
Bax R	CGGCTGAAGATTAGAGTTGT
Actin F	CATCAGGGTGTCATGGTTGGT
Actin R	TCTCTTGCTCTGAGCCTCATCA

manufacturer instructions. Briefly, 1 μg total mRNA of each sample was reverse-transcribed to obtain cDNA (Vazyme Biotech Co., Nanjing, China). The cDNA samples were diluted 10 times to be the final sample for qRT-PCR performed by the CFX Connect TM Real-Time system (Bio-Rad laboratories, Hercules, CA, USA) using specific primers (Table 1) equipped with SYBR green fluorescent dyes (Vazyme Biotech Co., Nanjing, China). Gene expression levels were normalized to the RNA expression of the housekeeping gene β-actin (relative quantification) with the △△CT correction (Song et al., 2018), as described earlier (Dodd et al., 2010).

2.5. Statistical analyses and data handling

All data are expressed as group means ± SEM, and analyzed using the unpaired Wilcoxon-Mann-Whitney *U* test test to compare the DM and control groups. *P* was set as 0.05 in all experiments. Sample size for each assay was based on previously published studies that used similar research approaches, our own pilot studies with the present zebrafish DM model, as well as based on statistical power analyses using online calculators. Recognizing the importance of biomedical data reproducibility (Masca et al., 2015), steps were taken to ensure data randomization and blinding. All animals were randomly allocated to the treatment groups using the online random number generator. The animal cohorts were housed in identical conditions (as described above), except for diet. While masking was not performed during animal care, the experimenters were blinded to the treatment groups during testing. Behavioral analyses were performed by two highly-trained scorers (intra/inter-rater reliability > 0.85, as assessed by Spearman correlation) blinded to the treatment. Biochemical analyses were also performed by experimenters blinded to treatment, aware only of encoded fish numbers. Analyses of data were performed by experimenters offline without blinding, since all animals and samples used were included in analyses and the analysts had no ability to influence the data.

3. Results

The BMI was significantly higher in DM zebrafish (0.41 ± 0.018 vs. 0.36 ± 0.012, $P = 0.02$, *U* test, $n = 20$), indicating that high glucose-high cholesterol diet promoted obesity in this group. Fig. 2 shows that this diet also significantly increases total glucose, triglyceride, total cholesterol and LDL, but reduced HDL levels, generally consistent with clinical and rodent DM responses. The mRNA expression qRT-PCR analyses also revealed upregulated glucagon in the muscle tissue of DM fish (Fig. 2).

Fig. 3 shows higher anxiety-like behavior in DM fish, as assessed by reduced exploration in the NTT (fewer top entries and time in top, longer latency to enter the top) and the LDB (fewer light entries and time in light, longer latency to enter the light), as well as by the elevated whole-body cortisol and brain GR mRNA expression. Taken together, this suggests the global activation of the zebrafish HPI axis and anxiogenesis in DM zebrafish fed high-glucose/high-cholesterol diet.

The whole-body levels of IFN-γ and IL-4 were elevated in the DM group (Fig. 4), which also displayed higher brain mRNA expression of the genes encoding IL-6 and TNF-α (but not other cytokines tested), as well as CD11b, GFAP, BDNF, its receptors Trk B and P75, caspase-3, Bax, but not BCl-2.

4. Discussion

This study is the first in-depth analysis of peripheral and central inflammation in zebrafish model of DM-evoked affective pathogenesis. Strengthening the model, the rise of BMI in DM zebrafish suggests an obesity-like phenotype that strikingly parallels clinical obesity caused by high intake of glucose and cholesterol (Buettner et al., 2012; Unger, 1991), and frequent clinical comorbidity between DM and obesity (Ouyang et al., 2014). The high-glucose/high-cholesterol diet in DM zebrafish also affected whole-body levels of glucose, triglyceride, total cholesterol, HDL and LDL (Fig. 2), resembling clinical T2DM with hyperglycemia and aberrant glucagon and gluconeogenesis (Clemens and Siegel, 2004). Increased glucagon mRNA levels observed here (Fig. 2) further support DM-like metabolic deficits in fish evoked by chronic high-glucose/high-cholesterol diet. Indeed, reduced sensitivity of the DM islet cells to glucose may upregulate glucagon, which can serve as a biomarker of hyperglycemia because high glucose consumption increases its secretion (Salehi et al., 2006). The expression of insulin and its receptor were unaltered in the DM fish despite robust hyperglycemia, suggesting deficits in insulin-glucagon signaling in the DM model (Fig. 2). Gluconeogenesis is common for noninsulin-dependent DM patients, and cortisol can increase gluconeogenesis in humans (Khani and Tayek, 2001).

Elevated anxiety-like behavior in both NTT and LDB (Fig. 3) further validates the present study, as it is in line with earlier behavioral findings in zebrafish DM models (Dos Santos et al., 2018) and supports the link between DM and affective pathogenesis (Lustman, 1988; Smith et al., 2013). On the one hand, anxiety behaviors can be evoked by inflammation caused by prolonged exposure to high-glucose/high-cholesterol diet (Chih-Yuan and Tien-Chun, 2004; De et al., 2006; Stuart and Baune, 2012). On the other hand, cholesterol is a precursor of cortisol, and its high intake may also promote cortisol synthesis, thereby contributing to elevated stress reactivity in DM fish observed here.

In the present study, higher IFN-γ levels suggest activated TH1 cells, whereas elevated IL-4 levels indicate activated TH2 cells (Fig. 4), see (Zhu et al., 2012) for review. As inflammation may activate the

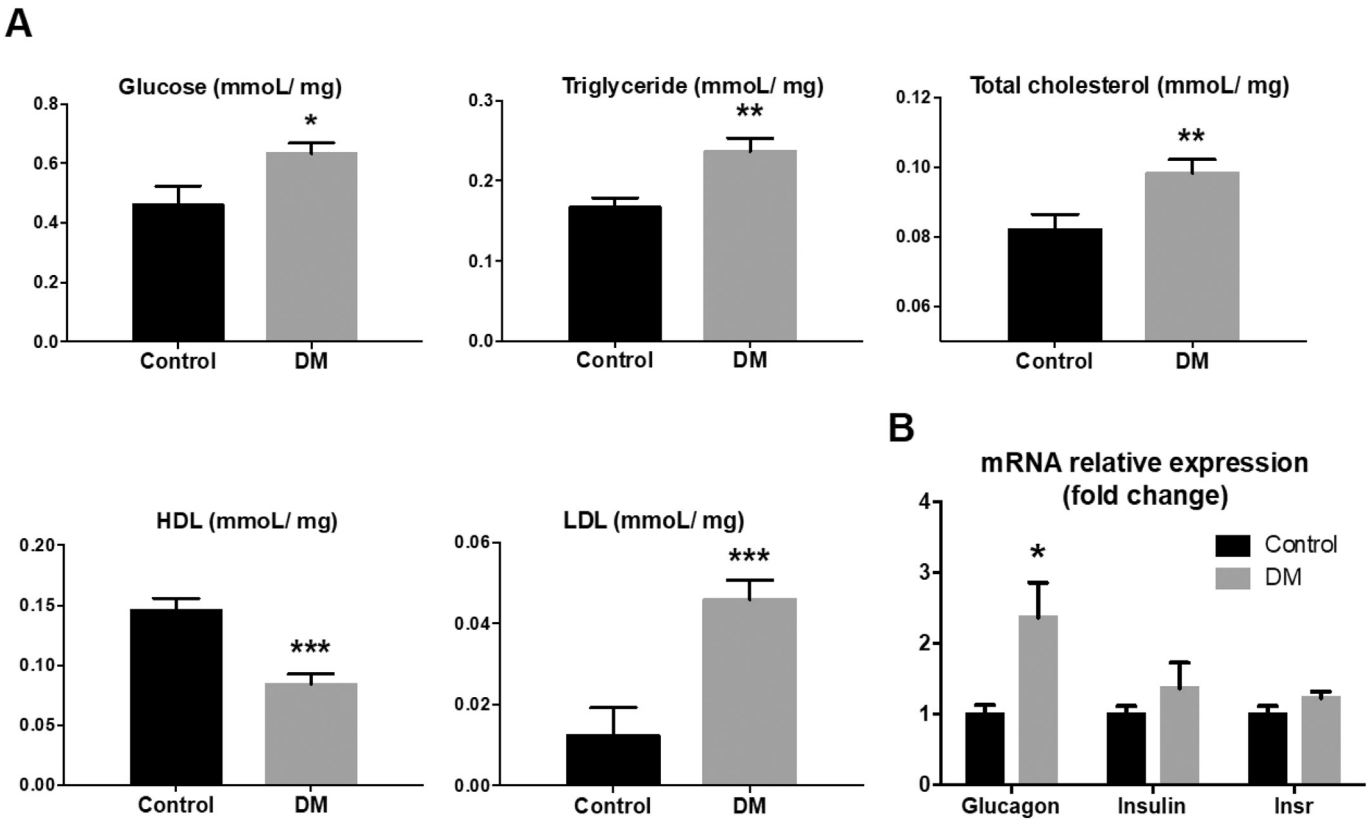

Fig. 2. Effect of high glucose/high cholesterol diet on whole-body biochemical parameters (A) and muscle tissue zebrafish DM-related genes' expression (B). *P < 0.05, **P < 0.01, ***P < 0.001, DM vs. controls groups, *U* test (n = 9–12 per group).

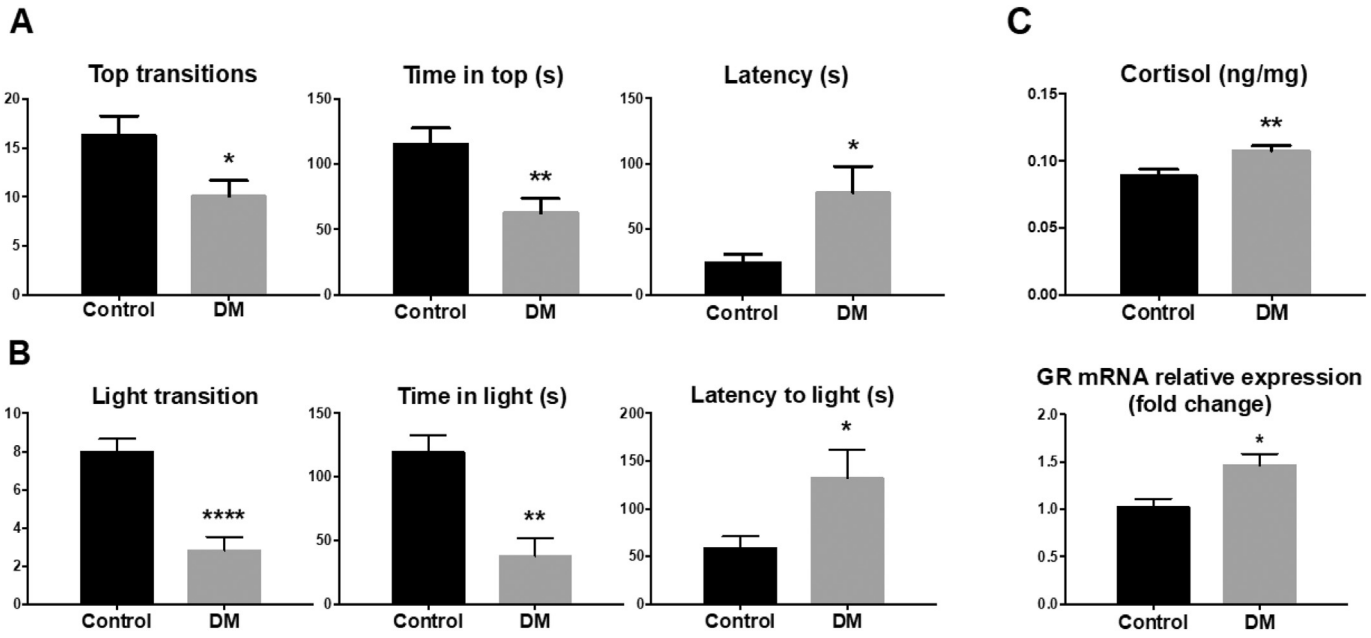

Fig. 3. Effects of high glucose/high cholesterol diet on zebrafish anxiety-like behavior in the novel tank test (A, n = 15–16 per group) and the light-dark box test (B, *n* = 13), as well as whole-body cortisol levels and the brain glucocorticoid receptor mRNA expression (C, *n* = 15 and 8, respectively). *P < 0.05, **P < 0.01, ****P < 0.0001, DM vs. control groups, *U* test.

neuroendocrine axis (Joseph and Golden, 2017), sustaining high levels of cortisol secreted by the zebrafish HPI axis may explain anxiety-like behavior observed in the present study (Fig. 3). Furthermore, both inflammation and stress may affect the permeability of the blood brain barrier, thus altering brain levels of neurotrophins and promoting neuroinflammation (Maddison and Chen, 2017). Indeed, both astrocytes and microglia were activated in the present study, given the up-regulation of their respective neuroglial markers GFAP and CD11b (Fig. 4). Paralleling microglial activation, the brain mRNA levels of key pro-inflammatory cytokines (IL-6 and TNF-α) also increased (Fig. 4), in line with some clinical and rodent DM and depression studies (Mirza et al., 2012; Szelachowska et al., 1998; Tsiavou et al., 2004). However,

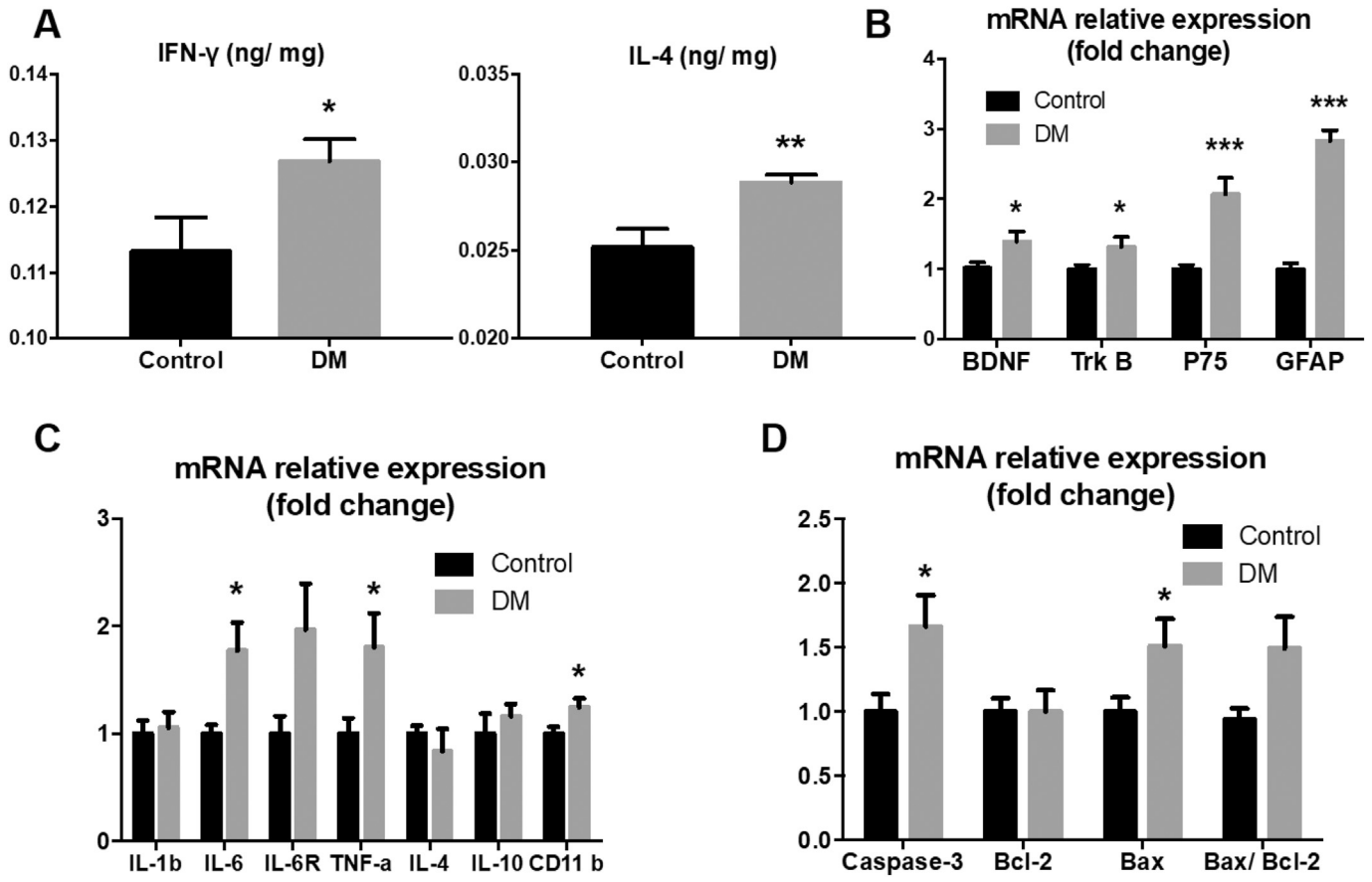

Fig. 4. Effects of high-glucose/high-cholesterol diet on peripheral inflammatory markers, a pro-inflammatory cytokine IFN-γ and an anti-inflammatory cytokine IL-4 (A, $n = 12$ per group) and the brain mRNA expression of BDNF and its receptors, GFAP (B, $n = 9$–12), selected cytokines (C, $n = 8$) and apoptosis genes (D, n = 9–12). *P < 0.05, **P < 0.01, ***$P < 0.001$, DM vs. control groups, U test.

BDNF was also elevated in zebrafish (Fig. 4), suggesting the greater neuroprotective ability of these fish (than mammals) in CNS disease, likely mediated by astrocytes and activated BDNF-TrkB signaling, both known to promote *neuroprotection*. In contrast, the BDNF action at its P75 receptor in-vivo promotes *apoptosis* (Anand and Mondal, 2018). Notably, both TrkB and P75 mRNA expression increased in the present study (Fig. 4), suggesting that both processes co-occurred in zebrafish DM model developed here. Finally, a significant upregulation of the two key apoptotic genes, *caspase-3* and *Bax*, in DM zebrafish in the present study (Fig. 4) supports the role of apoptotic mechanisms in DM-stress interplay (Chen et al., 2017).

Clearly, there are several limitations of the present study to be considered. For example, no established animal model of a disorder, yet alone a relatively new species like zebrafish, can fully recapitulate the clinical complexity of human DM and especially behavioral (affective) deficits (Kalueff et al., 2014b; Kalueff et al., 2012; Nguyen et al., 2013). We also recognize that the availability of modern gene-editing tools and genetically modified zebrafish may remarkably empower modeling DM-related pathologies in this organism. For example, several mutant or knockdown zebrafish models have already been reported in the literature, showing DM-like hyperglycemia, reduced beta cells and decreased insulin (Kimmel et al., 2015; Lodd et al., 2019). Thus, utilizing these genetic models to study the link between DM and affective pathogeneses may become a new promising line of research. However, as zebrafish underwent a teleost-specific genome duplication event, many fish genes have two copies of the human or rodent DM- and stress-related genes (Cachat et al., 2011; Fonseka et al., 2015; Maddison and Chen, 2017; Zang et al., 2017). This, in turn, may somewhat complicate direct translation of fish models into human responses.

Moreover, whole-body samples collected here differ from blood samples typically used to assess circulating cytokines in humans (Anderson et al., 1999; Mang et al., 2010; Mossop, 1983) and rodents (Fuliang et al., 2005; Song, 2015). Albeit much smaller than rodent brains, assaying zebrafish whole-brain samples used here may benefit from further region-specific analyses, likely to yield a more nuanced profile of their genomic responses to DM and stress. Given overt morphological abnormalities typically seen in DM, including in the brain and its vasculature, as well as in multiple other target organs, histological analyses of zebrafish brain and other tissues may be a valuable approach to generate morphological data to complement behavioral and physiological biomarkers assessed here. Likewise, zebrafish also possess some species differences (from mammals) in their metabolic physiology (Gut et al., 2017; Jurczyk et al., 2011; Schlegel and Gut, 2015), and also in behavioral, endocrine and immune responses (e.g., their ability to boost BDNF activity under stress (Song et al., 2018) rather than reduce it, as in rodents (Shen et al., 2014; Tolwani et al., 2002; Yu et al., 2015)). Finally, applying genetic and/or pharmacological therapies to rescue the DM-evoked behavioral and physiological deficits in zebrafish may be needed. Clearly, further in-depth analyses of these aspects will provide more insights into modeling the complex interplay between DM and affective pathogenesis in zebrafish.

In summary, chronic exposure to high-glucose/high-cholesterol diet evoked T2DM-like metabolic symptoms in zebrafish, accompanied by robust anxiety-like behavior, elevated cortisol, and altered central and peripheral cytokines, neurotrophin-related and apoptotic genes. Anxiety-like behaviors in DM zebrafish were accompanied by increased markers of peripheral inflammation, neuroinflammation and neuronal apoptosis. Taken together, these results link affective CNS pathogenesis in zebrafish to DM-evoked pathophysiological phenotypes, emphasizing the important role of metabolic deficits and their interplay with

neuroendocrine and neuroimmune mechanisms in affective disorders. Finally, given excellent potential of zebrafish for drug screening, our findings also suggest that high-glucose/high-cholesterol diet can be a simple protocol for inducing experimental T2DM and comorbid T2DM + anxiety in zebrafish. This approach may also have potential implications for modeling other metabolic disorders, their comorbid CNS conditions, as well as for searching novel anti-DM, anti-stress or 'combined' (anti-DM/anti-stress) drugs.

Ethical statement

The authors confirm no conflict of interest.

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