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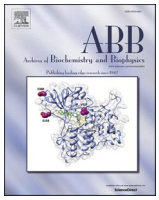

Simultaneous induction of systemic hyperglycaemia and stress impairs brain redox homeostasis in the adult zebrafish

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ABSTRACT

For diabetic patients it is crucial to constantly monitor blood glucose levels to mitigate complications due to hyperglycaemia, including neurological issues and cognitive impairments. This activity leads to psychological stress, called "diabetes distress," a problem for most patients living with diabetes. Diabetes distress can exacerbate the hyperglycaemia effects on brain and negatively impact the quality of life, but the underlying mechanisms remain poorly explored. We simulated diabetes distress in adult zebrafish by modelling hyperglycaemia, through exposure to dextrose solution, along with chronic unpredictable mild stress (CUMS), and evaluated brain redox homeostasis by assessing reactive oxygen species (ROS) content, the antioxidant system, and effects on mitochondrial biogenesis and fission/fusion processes. We also evaluated the total, cytosolic and nuclear content of nuclear factor erythroid 2-related factor 2 (NRF2), a critical regulator of redox balance, in the whole brain and total NRF2 in specific brain emotional areas. The combined CUMS + Dextrose challenge, but not the individual treatments, reduced total NRF2 levels in the entire brain, but strongly increased its levels in the nuclear fraction. Compensatory upregulation of antioxidant genes appeared inadequate to combat elevated levels of ROS, leading to lowering of the reduced glutathione content and total antioxidant capacity. CUMS + Dextrose treatment also upregulated transcription factors implicated in mitochondrial biogenesis and dynamics with a predominance of fission, which is consistent with increased oxidative stress. In conclusion, this study highlights the close interplay between hyperglycaemia and psychological distress causing overriding oxidative stress in the brain, rendering the organism vulnerable to the development of disease complications.

1. Introduction

Living with diabetes mellitus may entail considerable psychological burden, so much so that the prevalence of a phenomenon called "diabetes-related emotional distress" was first acknowledged as "separate from general emotional distress" and "an independent and major contributor to poor adherence" to the treatment regimen [1].

Distress due to diabetes proves to be an obstacle to the patient's therapeutic path, which includes frequent blood monitoring and management of hyperglycaemia, and medication adherence, worsening the overall quality of life [2–4]. The American Diabetes Association's "Standards of Care in Diabetes-2023 Abridged for Primary Care Providers" defines diabetes distress as a separate condition distinct from other comorbidities like depression and anxiety and urges that patients

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be assessed for it [5]. Despite this, diabetes distress and its involvement in the disease pathophysiology at molecular and cellular levels has not gained much attention. A valid argument for this could be that animal models do not allow the exclusive manifestation of diabetes distress Thus, studying psychological distress in diabetic conditions can only be carried out under the manifestation of chronic stress, which can be achieved by following the chronic unpredictable mild stress (CUMS) paradigm. Although there exist some challenges in model reproducibility and successful stress manifestation [6], the CUMS procedure is widely embraced to model mood disorders such as depression and anxiety [7,8], not only in rodents but also in the fish [9,10].

The zebrafish is a valuable disease model for exploring diabetes-related brain pathologies [11] as their pancreatic morphology is comparable to the mammalian pancreas, both sharing evolutionarily conserved developmental pathways for the insulin-producing beta cells in the islet of Langerhans [12]. Hyperglycaemia, the main diabetes marker, can be easily induced in fish via chronic exposure to standardized strengths of dextrose solution [13]. Fish exposed to dextrose are considered an adequate model of diabetes, as demonstrated by the impairment of their response to exogenous insulin [13]. Hyperglycaemic zebrafish exhibit behaviour abnormalities, disturbance in lipid metabolism, increased cortisol levels, blood-brain barrier dysfunction, neuroinflammation, and compromised neurogenesis [14–16].

A recurring consequence of the diabetic condition is redox imbalance [17], which involves the generation of high levels of reactive oxygen species (ROS) and inadequate antioxidative defence, a condition called oxidative stress in which increased oxidative damage to macromolecules and cellular dysfunction occurs [18]. Mitochondria, organelles involved in essential metabolic and respiratory processes, account for a significant part of cellular ROS generation and are their primary target [18]. ROS production and mitochondrial oxidative damage increase in oxidatively damaged mitochondria, which induces mitochondrial dysfunctions. Dysfunctional mitochondria exhibit dysregulation in mitochondrial vital processes such as biogenesis, fission/fusion, mitophagy, ATP production, and calcium homeostasis [19,20]. In diabetes, disturbances in mitochondrial processes occur in major physiological organs, including the pancreas and the brain [21].

An increasing body of scientific evidence indicates that a mediator of diabetes progression is the nuclear factor erythroid 2-related factor 2 (NRF2), a critical transcription factor that regulates redox, protein, and metabolic homeostasis. NRF2 is a basic leucine-zipper transcription factor that possesses the remarkable ability to regulate the expression of numerous phase II antioxidant response element (ARE)-dependent genes indispensable for central biological processes such as detoxification, cytoprotection, inflammation, and metabolism, among others [22]. While some studies support the beneficial effect of acute induction of NRF2 before the development of diabetic outcomes in most diabetes-relevant tissues [23], other studies indicate that NRF2 can play both a protective and pathogenic role depending on the timing and duration of activation [24,25]. Moreover, the involvement of NRF2 in the context of diabetes distress is still poorly explored. The main motive of this study is to bridge this gap by simultaneously modelling chronic hyperglycaemia and stress in adult zebrafish. In addition, we study the impacts of concurrent hyperglycaemia and stress on mitochondrial biogenesis and dynamics. This study aims to decipher whether concurrent distress accompanying hyperglycaemia is more detrimental to the brain when compared to the presence of either condition alone.

2. Materials and methods

2.1. Animals

Adult zebrafish (*Danio rerio*) of both sexes were used in the experiments. Fish were maintained under controlled temperature (26–28 °C) and photoperiod (14:10 h, light: dark), and given brine shrimp flakes twice a day (Ocean Nutrition Brine Shrimp Plus Flakes). Fish were allowed to acclimate to the facility environment for at least two weeks before the commencement of experiments. The guidelines of the EU Directive 2010/63 for the Care and Use of Laboratory Animals were followed in conducting this study. The Italian Minister of Health (Authorization No. 767/2023-PR.) and the University of Naples Federico II Committee on the Ethics of Animal Experiments authorized all animal procedures.

2.2. Induction of chronic unpredictable mild stress (CUMS) and hyperglycaemia

Fish were divided into four groups: Control, Dextrose, CUMS, and CUMS + Dextrose. Fig. 1 presents the experimental timeline of the study. Chronic hyperglycaemia was induced following the method of Dorsemans et al. [14], wherein fish were housed in tanks containing dextrose solution (111 mM). The solution was replaced daily to prevent the growth of harmful microorganisms and maintain constant dextrose exposure. To induce chronic stress, slight modifications were made to the method of Fulcher et al. [9]. As part of the CUMS paradigm, fish were subjected to seven different types of stressors: i) restraint (15 min), ii) overcrowding (60 min), iii) chasing with a net (8 min), iv) tank changes (six times in a row), v) elevation over water (2 min), vi) dorsal body exposure (2 min), and vii) isolation (60 min). The fish were exposed to two distinct stressors every day, randomly and unpredictably, for a total of 14 days.

2.3. Measurement of body weights and fasting blood glucose levels

On the 15th day, the body weights and fasting blood glucose levels of each individual fish were measured (n = 15 per group). Fish were briefly placed in dextrose-free clean water to wash away residual glucose on the outside of the body to avoid skewing of the blood glucose recordings. In addition, each fish was immediately wiped with absorbent tissue material to avoid blood dilution. For body weight measurements, they were anesthetized using the hypothermia method, which involved exposure to cold water measuring 10 °C. This was followed by euthanization employing deep hypothermic shock under ice (0–4 °C) followed by decapitation [13]. The fish were beheaded at the site of the pectoral girdle, rupturing the heart, after which a glucometer strip was delicately placed at the site of blood drainage [26]. The glucometer used was the Accu-Chek Instant S Blood Glucose Monitor. After recording the fasting blood glucose levels, whole brain samples were extracted and processed accordingly for further experiments.

2.4. Evaluation of oxidative stress

Following the assessment of fasting blood glucose levels, the brains were promptly extracted to prepare 10 % brain sample homogenate (in 0.1 M phosphate buffer at pH 7.4) for appropriate biochemical assays to estimate oxidative stress. The protein content of the samples in each group was estimated by the colorimetric Bio-Rad Bradford protein assay, using a commercial kit (Bio-Rad, Hercules, CA, USA).

Biochemical assays were performed to assess: i) reactive oxygen species (ROS) content, ii) total antioxidant capacity (TAC), iii) catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPX) activities, and iv) reduced glutathione (GSH) content, in the whole brains of zebrafish. For every biological replicate (n = 3), 5 whole zebrafish brains were pooled.

2.5. ROS content

ROS content was determined based on ROS-mediated conversion of 2′,7′-dichloro dihydro fluorescin diacetate (DCFH-DA) to the fluorescent dichlorofluorescein (DCF) [27]. Brain homogenate (25 μg of protein) was incubated in monobasic phosphate buffer (0.1 M, pH 7.4), supplemented with 10 μM DCFH-DA. After 15 min, 100 μM $FeCl_3$ was added,

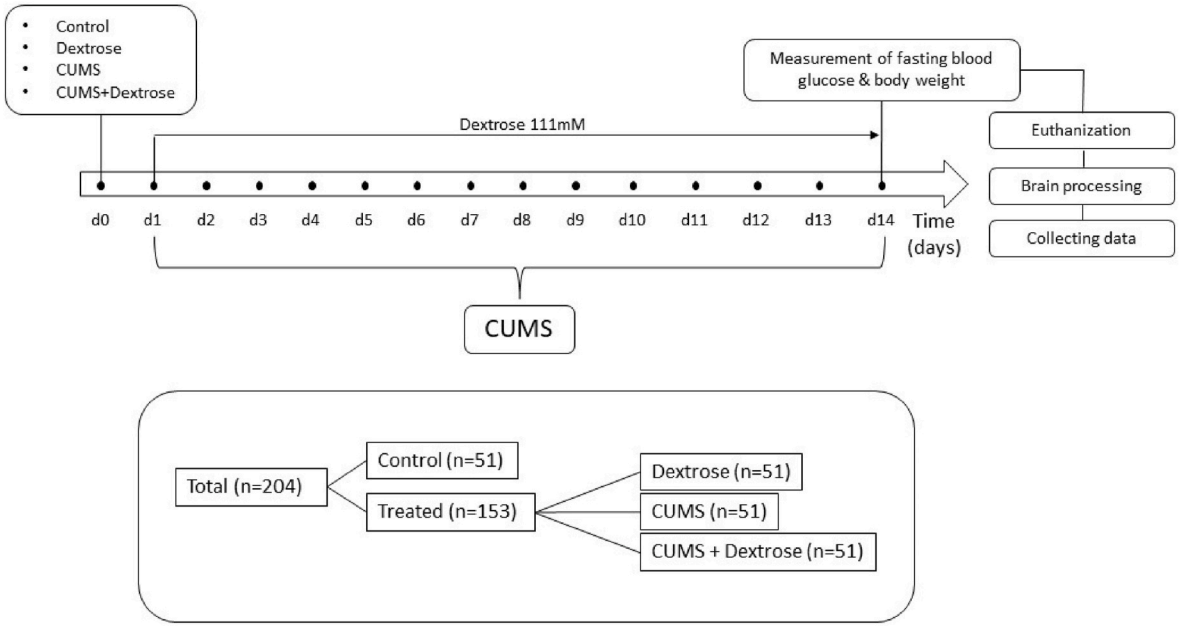

Fig. 1. Experimental design and timeline of the study. Fish were divided into the four experimental groups of control, Dextrose, CUMS, and CUMS + Dextrose. The Dextrose and CUMS groups were exposed to 111 mM dextrose solution and the CUMS procedure, respectively, for 14 days. At the end of the 14-day treatment, fasting blood glucose levels and body weights were measured, followed by whole brain sample extraction for downstream experiments. CUMS, chronic unpredictable mild stress.

and the mixture was incubated for 30 min. The conversion of DCFH-DA to the fluorescent product DCF was measured with excitation and emission wavelengths of 485 and 530 nm, respectively. To correct for background fluorescence, the conversion of DCFH to DCF was quantified in the absence of homogenate. The results were expressed as Relative Fluorescent Unit (RFU)/μg protein.

2.6. Total antioxidant capacities (TAC)

The TAC of brain tissue (using brain homogenate containing 0.01 mg of protein) was determined by measuring the decolorization of the mono-cation radical of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) at 734 nm, produced by the oxidation of 7 mM of ABTS induced by 245 mM of potassium persulfate [28]. The calibration curve was determined using 3,5-Di-*tert*-4-butylhydroxytoluene (BHT), and the results were expressed as BHT equivalents/mg protein.

2.7. Antioxidant enzyme activities in the whole brain homogenate

Catalase activity was evaluated according to Claiborne [29]. 25 μL of 10 % homogenate was added to 0.1 M phosphate buffer (pH 7.4) and 0.05 M H_2O_2. The reduction in the absorbance of H_2O_2 was kinetically followed at 240 nm. Catalase activity was calculated by using the molar extinction coefficient of H_2O_2 (39.6 M^{-1} cm^{-1}), and the results were expressed as μmol of H_2O_2 consumed per minute per milligram protein.

SOD activity was evaluated using the method of Marklund and Marklund [30], based on the inhibition of pyrogallol autoxidation by SOD, with some modifications. To 3 μl of 12.5 % triton-X-100, 15 μL of the of the 10 % homogenate was added. The resultant mixture was left to stand at 4 °C for 30 min. 90 μL (10.2 mg pyrogallol + 10 ml double distilled water + 8.6 μl HCl) of pyrogallol solution, 49.5 μL of 3 mM EDTA (pH 8), 750 μL of 0.1 M phosphate buffer, and 595.5 μL of double distilled water were combined to create the final assay solution. After gently blending the mixture, the absorbance was kinetically measured at 420 nm. SOD activity was presented as μmol of pyrogallol protected from oxidation per minute per milligram protein, using the molar extinction coefficient of 800 $\bullet 10^3$ M^{-1} cm^{-1}.

The activity of GPX was evaluated by following, at 340 nm, the lowering of the NADPH (1.5 mM) content due to the reconversion, catalysed by glutathione reductase (GR, 2.4 U mL^{-1}), of the GSSG produced by the oxidation of GSH (10 mM) in the reduction reaction of H_2O_2 (1.5 mM) by the GPX of 0.01 mg of whole brain homogenate protein [31]. The results were expressed as nmol NADPH oxidized/min • mg protein.

2.8. GSH content

Ellman's reagent (DTNB) was used to colorimetrically measure the total acid-soluble sulfhydryl concentrations to estimate reduced GSH levels with minor modifications to the method of Jollow et al. [32]. A volume of a solution of 4 % sulphosalicyclic acid was added to a volume 10 % brain homogenate, incubated at 4 °C for an hour and then centrifuged at 4000 rpm for 15 min. 200 μL of the supernatant was added to 1.1 mL of 0.1 M phosphate buffer (pH 7.4), followed by the addition of 200 μl of DTNB (10 mM). The absorbance was recorded at 412 nm and results were expressed as μ moles of GSH per gram of tissue utilizing the molar extinction coefficient of 1.36 • 10^4 M^{-1} cm^{-1}.

2.9. Quantitative Real-time Polymerase chain reaction (qRT-PCR)

Total RNA was isolated from homogenized brain tissue with 3 whole brains pooled for each biological replicate (n = 3) using the GeneJET RNA purification kit (Thermo Fischer Scientific, USA) in accordance with the manufacturer's instructions. RNA quantification was performed using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). cDNA was prepared from 20 ng of total RNA with the aid of the Verso cDNA synthesis kit (Thermo Fischer Scientific, USA). PowerUp™ SYBR Green master mix (Applied Biosystems, USA) was used for quantitative PCR assays, according to the manufacturer's instructions on a CFX Connect Detection System (Bio-Rad, Hercules, CA, USA). The qRT-PCR cycling protocol consisted of initial heating at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s. To confirm the specificity of the reaction, a dissociation curve analysis step was added after the thermal cycling protocol,

starting at 65 °C and ending at 95 °C, which included temperature increments of 0.5 °C every 5 s. A single melt peak was observed for all samples. *β-actin* was utilized as the endogenous control for normalization of the mRNA fold change in the different experimental groups. The primer sequences for the genes assessed have been provided in Table 1. The 2-ΔΔCT method was utilized to assess the relative mRNA expression levels of the investigated target genes.

2.10. Western blotting

Immunoblotting was performed as previously reported [33]. For each biological replicate (n = 3), five whole zebrafish brains were pooled. Briefly, the brains were homogenized in 300 μL of RIPA buffer (150 mM NaCl, 1.0 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris, pH 8.0), to which antiprotease cocktail (Merck, Life Science S.r.l., Milano, Italy) was added.

Nuclear and cytosolic fractions were obtained according to Ghanim et al., [34]. Briefly, the pool of five brains for each biological replicates (n = 3) were gently homogenized in a cytosolic extraction buffer (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, antiprotease cocktail (Merck, Life Science S.r.l., Milano, Italy), and 0.3 % Nonidet-P40, pH 7.9), and after 2 h of agitation at 4 °C, were centrifuged for 20 min at 16, 000g (4 °C) to obtain the cytosolic extract. The pellets were suspended in an ice-cold nuclear extraction buffer (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 20 % glycerol (v/v %), and antiprotease cocktail (Merck, Life Science S.r.l., Milano, Italy), pH 7.9) and after 2 h of agitation at 4 °C, were centrifuged for 5 min at 22,000g (4 °C) to obtain the nuclear fraction.

Protein content of each extract was quantified using the colorimetric Bio-Rad Bradford protein assay, using a commercial kit (Bio-Rad, Hercules, CA, USA). 40–80 μg of protein lysate was resolved in an SDS-PAGE gel and transferred onto a PVDF membrane. The blots were blocked with a blocking solution (5 % bovine serum albumin) for 1.5 h at room temperature. The blots were then incubated with the following commercially available primary antibodies: Dynamin-related protein 1 (DRP1) (1:1000, sc-271583, Santa Cruz, San Diego, CA, USA), Mitofusin-1 (MFN1) (1:1000, sc-166644, Santa Cruz, San Diego, CA, USA), peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC1-α) (1:1000, sc-518025, Santa Cruz, San Diego, CA, USA), nuclear respiratory factor 1 (NRF1) (1:1000, sc-33771, Santa Cruz, San Diego, CA, USA), nuclear respiratory factor 2/GA-binding protein alpha (NRF2/GABP-α) (1:1000, sc-22810, Santa Cruz, San Diego, CA, USA) and nuclear factor erythroid 2-related factor 2 (NRF2) (1:500, PA5-27882, ThermoFisher Scientific). As housekeeping proteins beta-actin (β-actin) (1:1000, sc-47778, Santa Cruz, San Diego, CA, USA) for cytosolic proteins, and Histone H1.0 (1:1000, E-AB-62092, ElabScience, Microtech, Naples, Italy) for nuclear proteins were used. Secondary antibodies were purchased from Santa Cruz (1:5000, anti-rabbit sc-2030, anti-mouse sc-525409, Santa Cruz, San Diego, CA, USA). After incubating the blots in primary antibodies overnight at 4 °C, they went through washing steps and were then incubated in the secondary antibody for 1 h. Bands were visualized using the excellent chemiluminescent detection Kit (ElabScience, Microtech, Naples, Italy), according to the manufacturer's instructions. Quantitative band densitometry was performed by analyzing ChemiDoc images.

2.11. Immunofluorescence

Immunostaining was performed following the protocol of Anand and Mondal [35] on coronal sections of the adult zebrafish telencephalon, utilizing three biological replicates (n = 3). The heads were fixed using 10 % formalin overnight at 4 °C, after which the fixed heads were washed twice with phosphate-buffered saline (PBS) followed by intact brain extraction. This was followed by fixing the extracted brains in 100 % methanol for a minimum duration of 16 h at −20 °C. Brains were then subjected to rehydration utilizing 75 %, 50 %, and 25 % of methanol gradations, followed by embedding the rehydrated brains in 2 % agarose. Free-floating coronal sections of 20 μm thickness were obtained with the help a vibratome (Leica VT-1200S). The sections were first incubated for 1 h at room temperature in blocking solution (0.2 % bovine serum albumin), and then overnight at 4 °C in rabbit polyclonal anti-NRF2 antibody (1:500, PA5-27882, ThermoFisher). The sections were then washed and incubated for 2 h at room temperature with goat anti-rabbit Alexa fluor-488 conjugated secondary antibody (1:1000; ab150077, Abcam, USA). After giving the sections another wash, 4′, 6-diamidino-2-phenylindole (300 nM DAPI, SRL chemicals, India) was used for counterstaining. The sections were mounted on microscopy slides with the help of a fluoromount aqueous mounting media (Fluoromount-G, Southern Biotech), and imaged at 10X and 60X magnifications using a confocal microscope (Nikon A1R confocal).

2.12. Statistical analysis

The quantified data are expressed as the mean ± standard error of the mean (SEM). To perform statistical analysis, GraphPad Prism software version 9 was used. Normal distribution and equal variance of the data were evaluated by Shapiro-Wilk test and Brown Forsythe test, respectively. One way analysis of variance followed by Tukey post-hoc test was applied for normally distributed data. When equal variance was not given, Kruskal-Wallis nonparametric test followed by Dunn's post-test was performed. The p-values of *p < 0.5, **p < 0.01, ***p < 0.001, and ****p < 0.0001 were assigned to indicate statistical

Table 1
Specific primer pairs used for qRT-PCR assays.

	Sense Primer (5′–3′)	Antisense Primer (5′–3′)
1. nrf2	*AACGAGTTCTCCCTTCAGCA*	*ATTTTGTCGCCGATTTTGTC*
2. keap1	*TGGATAACTACCTCTATGCCGT*	*CCTTGGTTAAATCCACCTAACAC*
3. sod1	*CAATGCTAACTTTGTCAGGCCA*	*CCTTCCCCAAGTCATCCTCC*
4. sod2	*CTTGGGATAGATGTCTGGG*	*GTGGTCTGATTAATTGTGCG*
5. catalase	*AACCAACAACCCTCCAGACAG*	*TCCGCTCTCGGTCAAAATGG*
6. gpx	*AGATGTCATTCCTGCACACG*	*AAGGAGAAGCTTCCTCAGCC*
7. gclc	*AACCGACACCCAAAGATTCAGCACT*	*CCATCATCCTCTGGAAACACCTCC*
8. pgc-1α	*ACCAACCATCTTGCCACTTC*	*ATTACTCAGCCTGGGCCTTT*
9. nrf1	*AGGCCCTGAGGACTATCGTT*	*GCTCCAGTGCCAACCTGTAT*
10. nrf2/gabpb1	*AAGTGGACCGAACACCCCTG*	*CTTGCTGAGCGCGTGAACAT*
11. mfn1	*CTGGGTCCCGTCAACGCCAA*	*ACTGAACCACCGCTGGGGCT*
12. dnm1l/drp1	*CGATCCTCTTGGCGGACTTACA*	*AATAGAGCAGGACGCGGACC*
13. β-actin	*CGAGCAGGAGATGGGAACC*	*CAACGGAAACGCTCATTGC*

nrf2, nuclear factor erythroid 2-related factor 2; *keap1*, Kelch-like ECH-associated protein 1; *sod1*, superoxide dismutase 1; *sod2,* superoxide dismutase 2; *gpx,* glutathione peroxidase; *gclc,* glutamate-cysteine ligase catalytic subunit; *pgc-1α*, aperoxisome proliferator-activated receptor-gamma coactivator-1 alpha; *nrf1,* nuclear respiratory factor 1; *nrf2/gabpb1,* nuclear respiratory factor 2/GA-binding protein transcription factor subunit beta 1; *mfn1,* mitofusin 1; *dnm1l/drp1,* dynamin 1-like/dynamin-related protein 1; *β-actin,* beta-actin.

significance between the different experimental groups.

3. Data Availability

All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

4. Results

4.1. CUMS + Dextrose treatment induced hyperglycaemia but did not alter body weights

Fasting blood glucose levels (Fig. 2a) were elevated in animals exposed to the dextrose solution, but not in the groups exposed to CUMS. Body weights (Fig. 2b) were not significantly affected by treatments.

These results agree with the observation that such a protocol induces a 3-fold increase in fasting blood glucose levels without producing changes in body weight [36–38].

To assess if treatments affected brain redox status, we measured ROS content, total antioxidant capacity, GSH levels, and the mRNA content of the catalytic subunit of glutamate-cysteine ligase (*gclc*), the first rate-limiting enzyme in glutathione synthesis.

4.2. CUMS + Dextrose treatment resulted in increased ROS content and reduced TAC in the brain

ROS levels (Fig. 3 a) were significantly increased in the CUMS, and CUMS + Dextrose groups when compared to the control, suggesting that the increase can be due to the CUMS treatment. TAC levels (Fig. 3 b) were significantly lowered in the CUMS + Dextrose treatment group. Since neither Dextrose nor CUMS alone show effects on TAC levels, we can hypothesize that the combination of treatments has a synergistic effect.

GSH content (Fig. 3c) was significantly reduced in all three treatment groups, and the *gclc* mRNA content (Fig. 3d) was not significantly different among groups. These results indicate that when hyperglycaemia is associated with stress, it can lead to an imbalance between the production and the tissue's ability to cope with ROS.

To obtain more information on the effects of the treatments on the antioxidant defence system, we evaluated the activity and mRNA content of some of the main components of the antioxidant enzymatic system (SOD, catalase and GPX).

4.3. CUMS + Dextrose treatment affected the antioxidant enzyme system

All treatments induced a significant increase in SOD activity (Fig. 4a), with the CUMS group showing the lowest increase. In the CUMS + Dextrose group, the increase was not significantly different from that observed in the Dextrose group which suggests a predominant effect of the Dextrose treatment. These changes were associated with increases in the mRNA levels of *sod1* for the CUMS and CUMS + Dextrose groups (Fig. 4b), and of *sod2* (Fig. 4c) in the CUMS + Dextrose group, although a tendency towards the increase was also observed in the Dextrose and CUMS groups.

GPX activity (Fig. 4d) was significantly boosted in the CUMS and CUMS + Dextrose groups with the highest increase in the CUMS + Dextrose group, suggesting a synergistic action of the two treatments. The levels of the *gpx* mRNA (Fig. 4e) were unaffected by treatments, although a tendency towards the increase was observed in all treatment groups.

Catalase activity (Fig. 4f) increased in the CUMS + Dextrose group, indicating a synergistic activity of Dextrose and CUMS, while *catalase* mRNA levels (Fig. 4 g) did not change in all three treatment groups, although they showed a tendency towards the increase with all treatments.

To obtain information on the effects of treatments on NRF2, the ubiquitous master transcription factor that upregulates gene expression of antioxidant enzymes, we determined the treatments' effect on its protein and mRNA content, and on the mRNA content of its main negative regulator Kelch-like ECH-associated protein 1 (KEAP1).

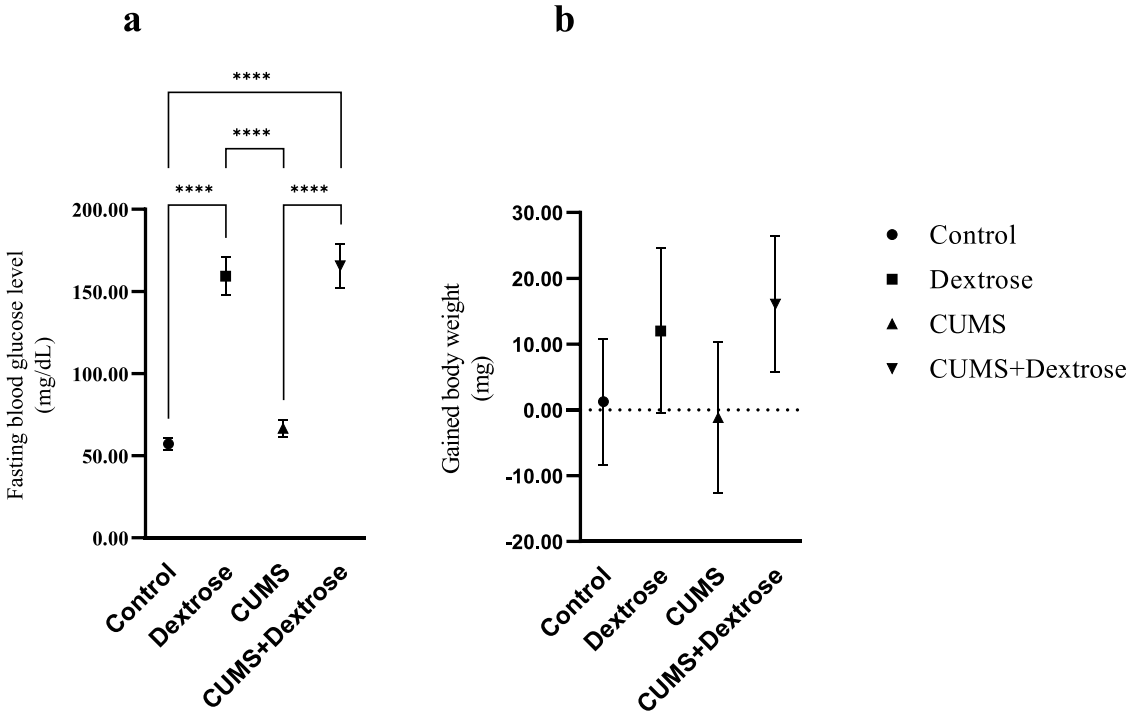

Fig. 2. Effect of Dextrose, CUMS, and CUMS + Dextrose treatment on fasting blood glucose levels (a) and gained body weight (b). Data presented is the average of fifteen determinations obtained from fifteen different animals. ***, $p < 0.001$) ****, $p < 0.0001$). Each value is represented as mean ± SEM (n = 15).

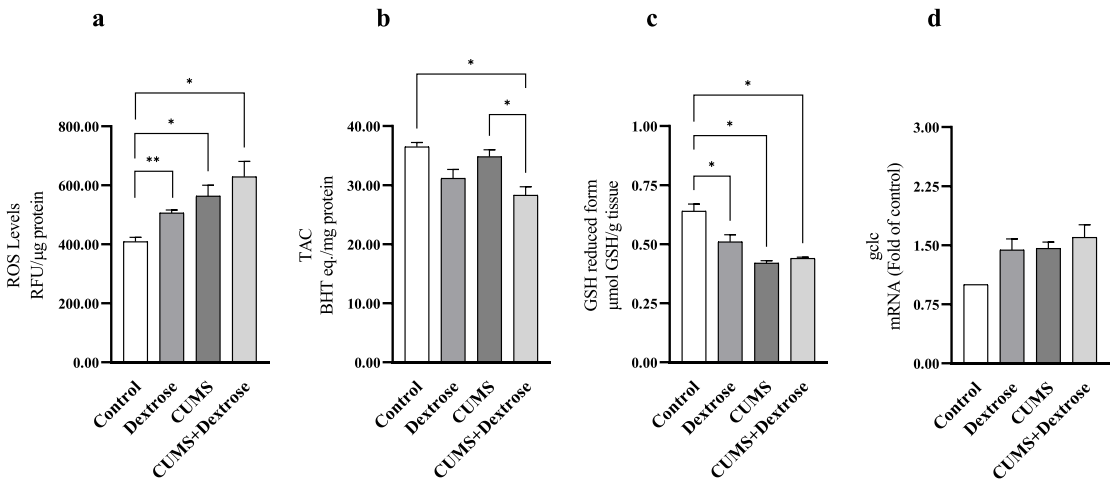

Fig. 3. Effect of Dextrose, CUMS, and CUMS + Dextrose treatment on ROS content (a) and total antioxidant capacities (TAC) (b), GSH content (c), relative mRNA quantification of indicated gene: *gclc* (d). Nonparametric test was used for *gclc* mRNA content (panel d) statistical analysis. Data presented is the average of three determinations of different homogenates obtained from three-five pooled zebrafish brains. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Each value is represented as mean ± SEM (n = 3).

Furthermore, by immunofluorescence, we evaluated the distribution of the protein in the telencephalon of animals subjected to the various treatments.

4.4. CUMS + Dextrose treatment reduced total NRF2 content but increased the nuclear fraction in the brain

The NRF2 protein content in the whole brain tissue (Fig. 5 a, b), was significantly reduced only in the CUMS + Dextrose group which suggests that the combination of Dextrose and CUMS treatments exerts a synergistic effect. By detecting the NRF2 protein content in cytosolic and nuclear fractions, we observed that all treatments reduced the cytosolic (Fig. 5 a, c) and increased the nuclear NRF2 content (Fig. 5 a, d). The changes in both fractions were more pronounced in the CUMS + Dextrose group further supporting the idea that the combination of hyperglycaemia and emotional stress have synergistic action.

Interestingly, the mRNA levels of *nrf2* (Fig. 5 e) were significantly increased in CUMS and CUMS + Dextrose groups, with a tendency to the increase in the Dextrose group. The mRNA content of *keap1* (Fig. 5 f) significantly increased in the same extent in Dextrose and CUMS + Dextrose groups.

Furthermore, the NRF2 protein content was significantly lowered in CUMS and CUMS + Dextrose groups in the medial zone of the dorsal telencephalic area (Dm) and parts of the dorsal nucleus of the ventral telencephalic area (Vd) in the telencephalic region of the brain (Fig. 6 a, b), suggesting a crucial role of emotional stress in NRF2 reduction.

Since the mitochondrial population can undergo alterations in conditions of altered redox balance, we analysed the effects of the treatments on mitochondrial biogenesis and dynamics by evaluating the protein and mRNA content of factors involved in such processes. In particular, we assayed the protein and mRNA content of the master regulator of mitochondrial biogenesis, the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- α), the protein and mRNA content of the nuclear transcription factors activated by PGC1-α, the nuclear respiratory factor 1 (NRF1) and 2/GA-binding protein alpha (NRF2/GABP-α), and the protein and mRNA content of DRP1 and MFN1, protein markers of mitochondrial fission and fusion, respectively.

4.5. CUMS + Dextrose treatment prompted mitochondrial biogenesis, fission, and fusion in the brain

Protein levels of PGC1-α, NRF1 and NRF2/GABP-α (Fig. 7 a, b) were significantly increased in the CUMS and CUMS + Dextrose groups, suggesting a prevalent role for stress in inducing the increase in the factors involved in mitochondrial biogenesis. The treatments differently affected mRNA levels of the respective genes: *pgc-1α* mRNA was increased in CUMS, *nrf1* mRNA was increased in Dextrose and CUMS + Dextrose, and *nrf2/gabpb1* mRNA was increased in CUMS and CUMS + Dextrose (Fig. 7c).

Protein levels of the mitochondrial fission (DRP1) and fusion (MFN1) markers (Fig. 8 a, b), were significantly higher in both the CUMS and CUMS + Dextrose compared to the control and Dextrose groups. In the CUMS + Dextrose group, DRP1 protein content was also significantly higher than in CUMS, suggesting a possible synergistic effect of the two treatments. Both *dnm1l/drp1*and *mfn1* mRNA levels (Fig. 8c) were significantly increased in the CUMS and CUMS + Dextrose groups.

5. Discussion

In the present paper, we reported that hyperglycaemia affected brain redox homeostasis more extensively when associated with distress.

Oxidative stress is a common denominator of metabolic and neurobiological diseases, triggering and perpetuating damage to CNS functioning [17]. We found significant increases in ROS levels in the stressed fish independently from dextrose treatment and a reduced antioxidant capacity only when stress was associated with dextrose exposure. ROS content changes were consistent with previously published studies showing a CUMS-induced increase in ROS levels in zebrafish [39,40].

The reduced total antioxidant capacity observed only when CUMS regimen was associated with dextrose exposure suggested that the zebrafish brain was more susceptible to oxidative challenge when distress accompanied by hyperglycaemic conditions, even if a reduction of the levels of the reduced glutathione verified in all three treatment groups. The mRNA content of the glutamate-cysteine ligase catalytic subunit (*gclc*) appeared to increase, although this increase was not statistically significant.

The enzyme glutamate-cysteine ligase is a heterodimer constituted of the *gclc*, which possesses enzymatic activity, and the glutamate-cysteine ligase modifier subunit [41]. The catalytic subunit of the enzyme is sensitive to GSH which limits its synthesis [41]. The redox-sensitive

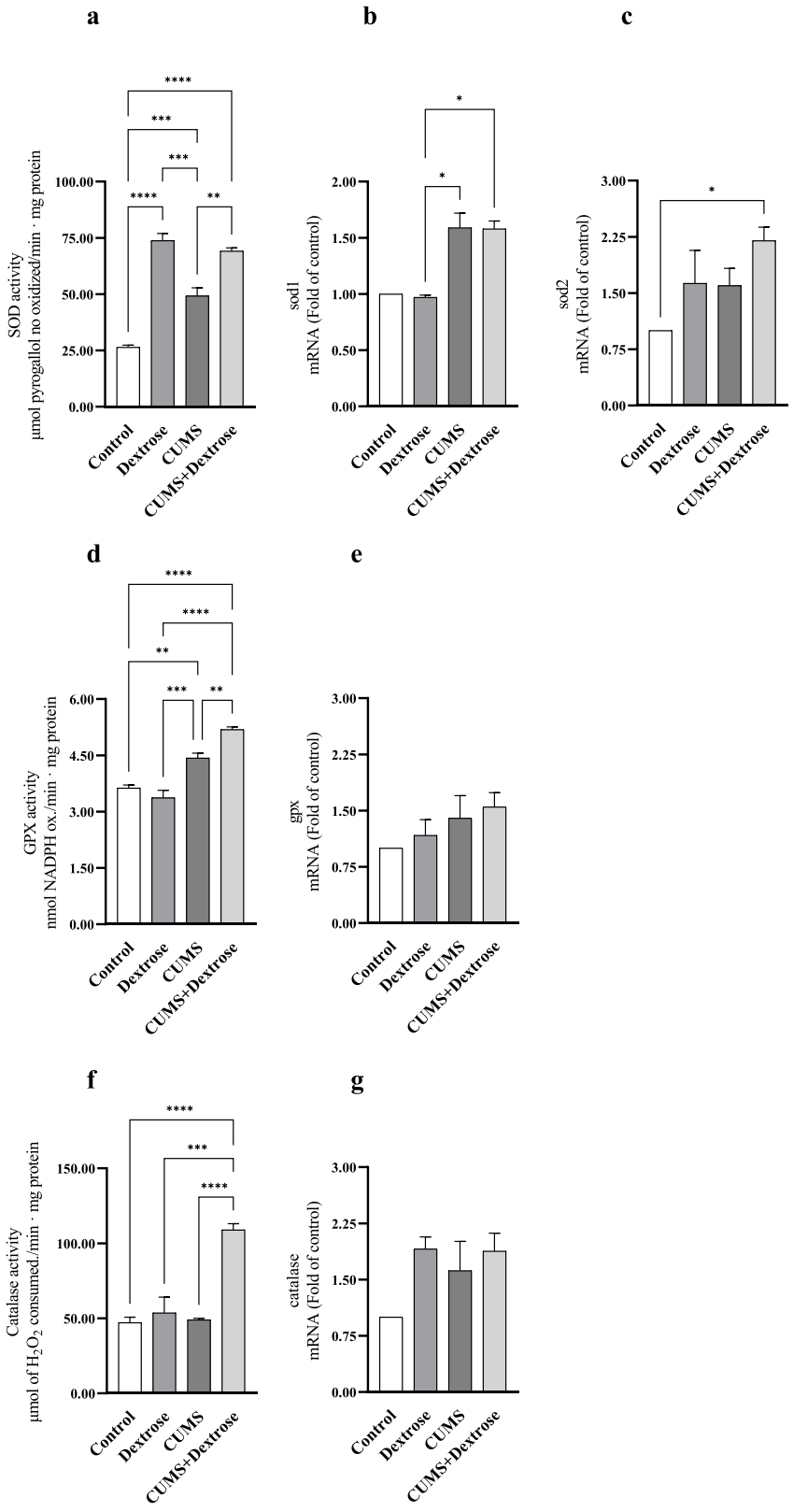

Fig. 4. Effect of Dextrose, CUMS, and CUMS + Dextrose treatment on SOD activity (a), relative mRNA quantification of indicated genes: *sod1* (b), and *sod2* (c), GPX enzyme activity (d), relative mRNA quantification of indicated gene: *gpx* (e), catalase activity (f), and relative mRNA quantification of indicated gene: *catalase* (g). Nonparametric test was used for *sod1, sod2, gpx*, and catalse mRNA content (panels b, c, e and g, respectively) statistical analysis. Data presented is the average of three determinations of different homogenates obtained from three-five pooled zebrafish brains. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) ****, $p < 0.0001$). Each value is represented as mean ± SEM ($n = 3$).

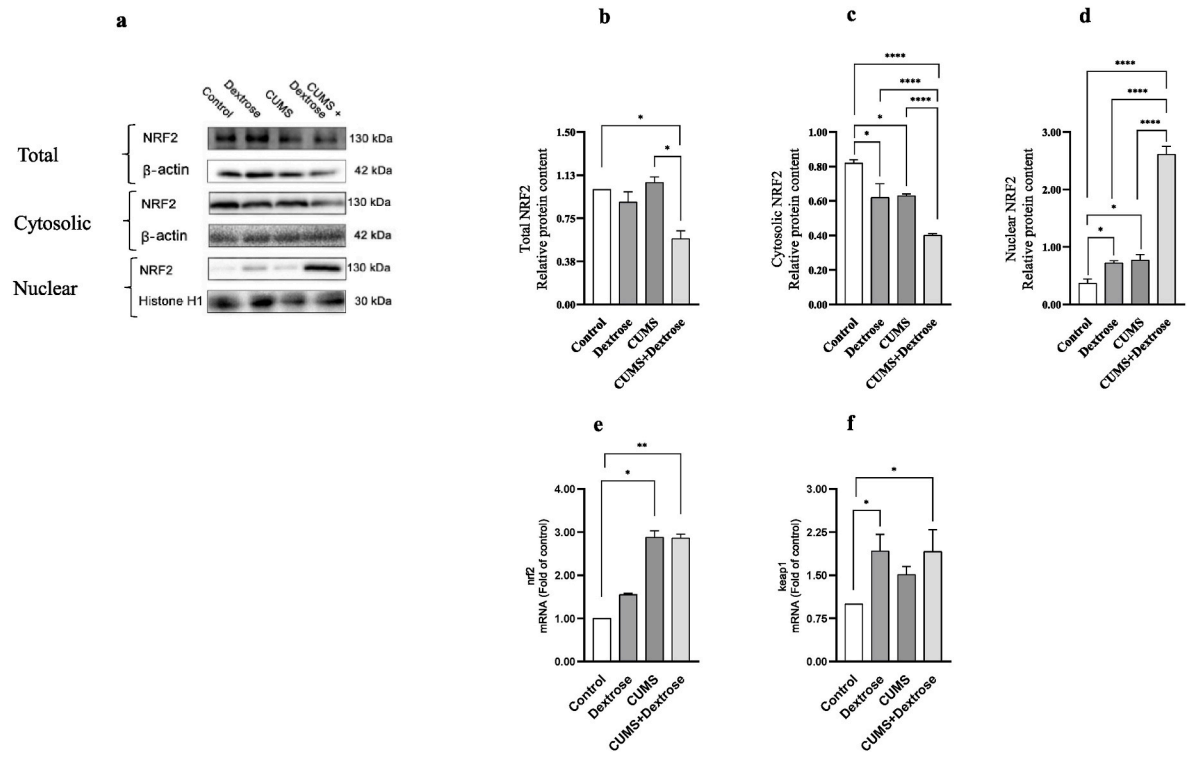

Fig. 5. Effect of Dextrose, CUMS, and CUMS + Dextrose treatment on total, cytosolic and nuclear NRF2 protein content. NRF2 representative western blots (a), total NRF2 relative protein content (b), cytosolic relative NRF2 protein content (c), nuclear relative NRF2 protein content and relative mRNA quantification of indicated genes: *nrf2* (e), and *keap1* (f). Nonparametric test was used for statistical analysis of mRNA content of *nrf2* and *keap1*. Data presented is the average of three determinations of different homogenates obtained from three-five pooled zebrafish brains. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) ****, $p < 0.0001$). Each value is represented as mean ± SEM. ($n = 3$).

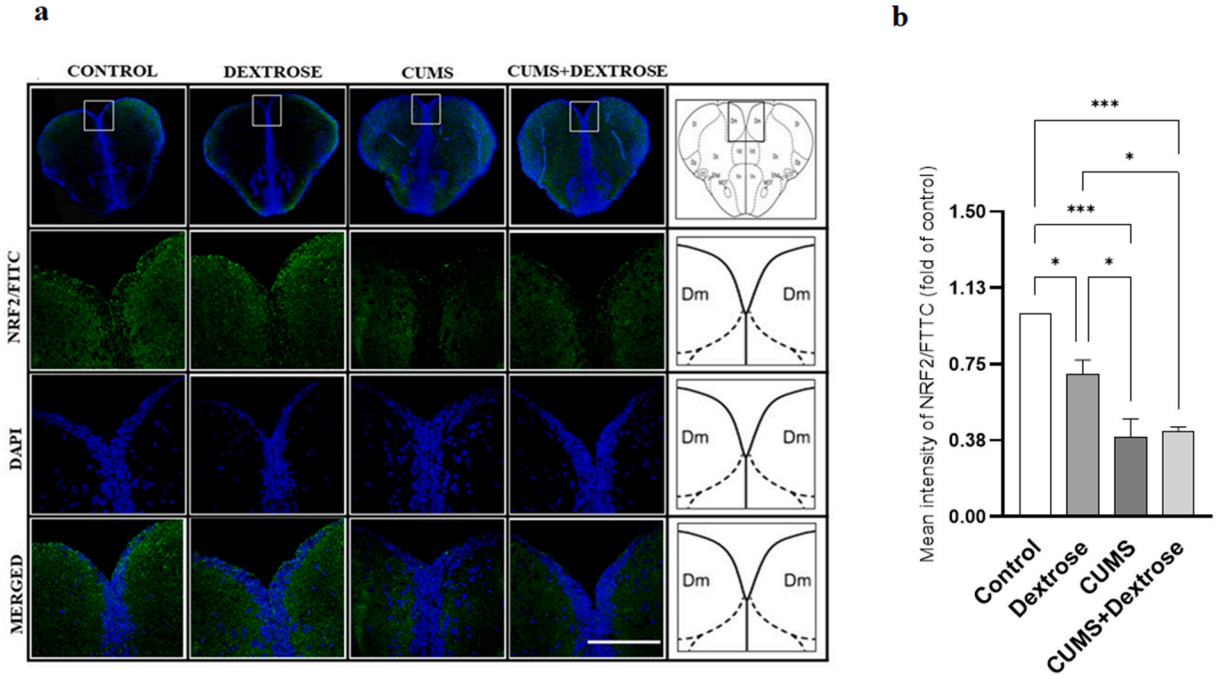

Fig. 6. Effect of Dextrose, CUMS, and CUMS + Dextrose treatment on NRF2 immunoreactivity in the telencephalon of adult zebrafish. Representative images showing immunostaining of NRF2 (green) counterstained with DAPI (blue) (a) and estimated mean fluorescence intensity of NRF2 (depicted as fold of control) (b). The scale bar indicates 100 μm. Dm: medial zone of dorsal telencephalic area; Vd: dorsal nucleus of ventral telencephalic area. Nonparametric test was used for statistical analysis of all parameters analysed. Data presented is the average of three determinations of different homogenates obtained from three-five pooled zebrafish brains. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) ****, $p < 0.0001$). Each value is represented as mean ± SEM. ($n = 3$).

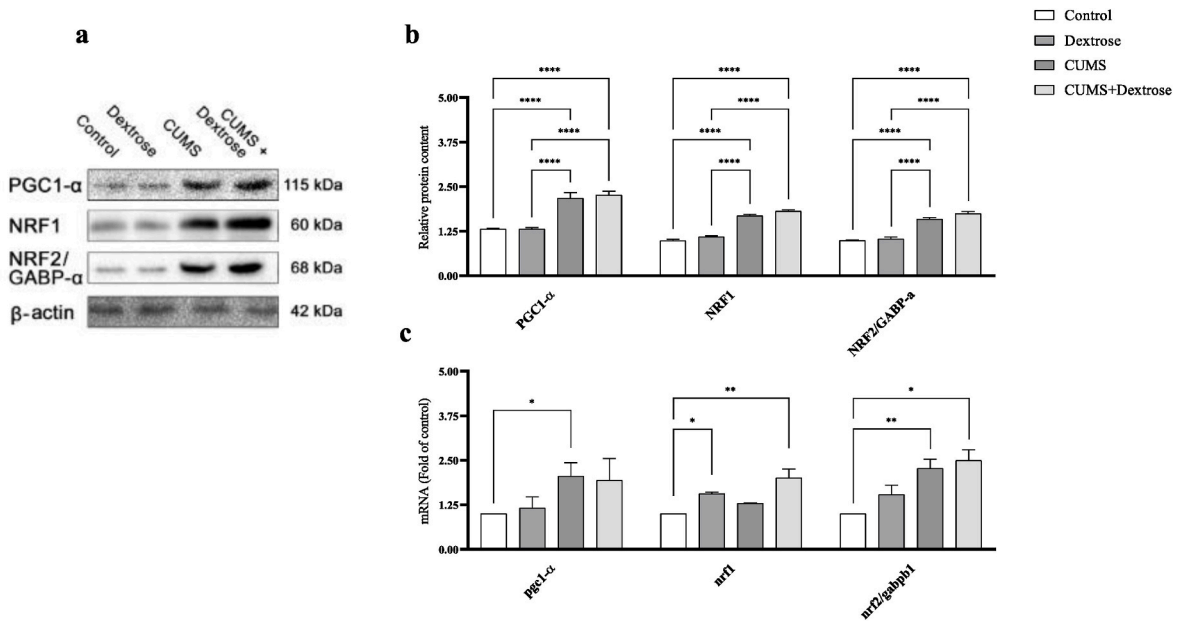

Fig. 7. Effect of Dextrose, CUMS, and CUMS + Dextrose treatment on mitochondrial biogenesis: PGC1-α, NRF1, NRF2/GABP-α representative western blots (a), and relative protein content (b), and relative mRNA quantification of indicated genes: *pgc1-α*, *nrf1*, and *nrf2/gabpb1* (c). Nonparametric test was used for *pgc1-α, nrf1* and *nrf2/gabpb1*, mRNA content (panel c) statistical analysis. Data presented is the average of three determinations of different homogenates obtained from three -five pooled zebrafish brains. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Each value is represented as mean ± SEM. (n = 3).

signalling pathway comprised of the nuclear factor erythroid 2-related factor 2 (NRF2)/electrophile response element (EpRE) system NRF2/EpRE [42] mediates *gclc* subunit gene expression. The nuclear localization of transcription factor NRF2, and the subsequent binding to a DNA recognition sequence known as an antioxidant response element (ARE) or EpRE [22], is considered an essential feature of the stress response.

After 14 days of treatments, the whole brain NRF2 protein content was significantly reduced when the hyperglycaemic stress was associated with distress (CUMS + Dextrose group). The mRNA content of *nrf2* in the total brain showed an increase in psychologically stressed fish independently from hyperglycaemia (CUMS and CUMS + Dextrose groups). In the dextrose group, the tendency toward the increase of the mRNA of *nrf2* agreed with the increase observed after longer exposure time (92 days) to dextrose [43], while its induction with emotional stressors agreed with the observation that increases in *nrf2* mRNA content were involved in the underlying mechanisms of some neurometabolic disorders [44].

We are not able to furnish a convincing explanation for the reduction in the brain content of NRF2 protein and the increase of its mRNA in the CUMS + Dextrose group. The complex regulation of NRF2 activity at multiple levels, including transcriptional, post-transcriptional, post-translational regulation, and regulation of NRF2 stability [45] can explain this picture. Furthermore, because both ageing and neurodegenerative diseases have been reported to be associated with increased oxidative stress and reduction in NRF2 content [46,47], we could hypothesize that in CUMS + Dextrose group, the processes that impair brain functions were accelerated. This explanation is, however, limited by the observation that the changes with age in the NRF2 content in the brain have also been reported to increase [48]. However, an increase has been reported after 6 months in the mice brain while a reduction has been reported in the mice spinal cord and astrocytes after 13 months [48], which suggests that the age-linked changes in NRF2 could be time-dependent as also found in the NRF2 changes in the neurodegenerative diseases [24].

We also found an upregulation of the mRNA content of the NRF2 repressor, *keap1*, in Dextrose and CUMS + Dextrose groups, according to the fact that NRF2 may stimulate *keap1* gene expression (increasing the mRNA content) for its own degradation. The negative feedback can limit the undue expression of NRF2 and uncontrolled NRF2 signalling [49].

The information derived from the analysis of NRF2 protein content in the whole brain furnished only a partial evaluation of the real impact of the treatments and did not give information on the relative content and localization of NRF2 in specific brain regions. Therefore, we evaluated by immunostaining the distribution of the protein in the telencephalon. All the treatments led to a reduction of NRF2 protein content in the medial zone of the dorsal telencephalic area (Dm) and a small part of the dorsal nucleus of the ventral telencephalic area (Vd), regions speculated to be comparable to the mammalian amygdala and basal ganglia, respectively [50,51]. Dm and Vd brain regions are heavily implicated in chronic stress, emotional regulation, fear, and anxiety [52–54] and are vulnerable to the ill effects of oxidative stress [55,56] in fish. The comparable reduction of NRF2 in Vd and Dm of CUMS fish brain, independently from dextrose exposure, agreed with similar results found in rat brains exposed to chronic isolation that induce anxiety and depressive-like behaviour [57], suggesting that mammalian and fish share common mechanisms involved in psychological stress development.

Interestingly, we also found a reduced content of NRF2 in the Dm and Vd brain regions of hyperglycaemic fish, which agreed, at least in part, with the observation that NRF2 was markedly inactivated in the brain of rats exhibiting diabetes-associated cognitive dysfunctions [58], further supporting the complex cellular regulation of the nuclear transcription factor [45]. On the other hand, our observation supports the idea that investigating the potential therapeutic qualities of NRF2 activators is an exciting opportunity to manage both diabetes and distress, two bidirectional and comorbid conditions [59].

The changes in the redox state due to the association of CUMS and dextrose treatments increased the activities of the enzymes SOD, GPX and catalase, with a significant increase also of *sod1* and *sod2* mRNA content. The CUMS treatment alone induced an increase in SOD and GPX activity with increased *sod1* mRNA content, while dextrose treatment alone significantly affected only SOD activity. These results agree with the changes in the relative distribution of NRF2 among cytosol and nucleus. Indeed, in the nuclear fraction of CUMS + Dextrose we found a more pronounced increase of NRF2 with respect to CUMS and Dextrose

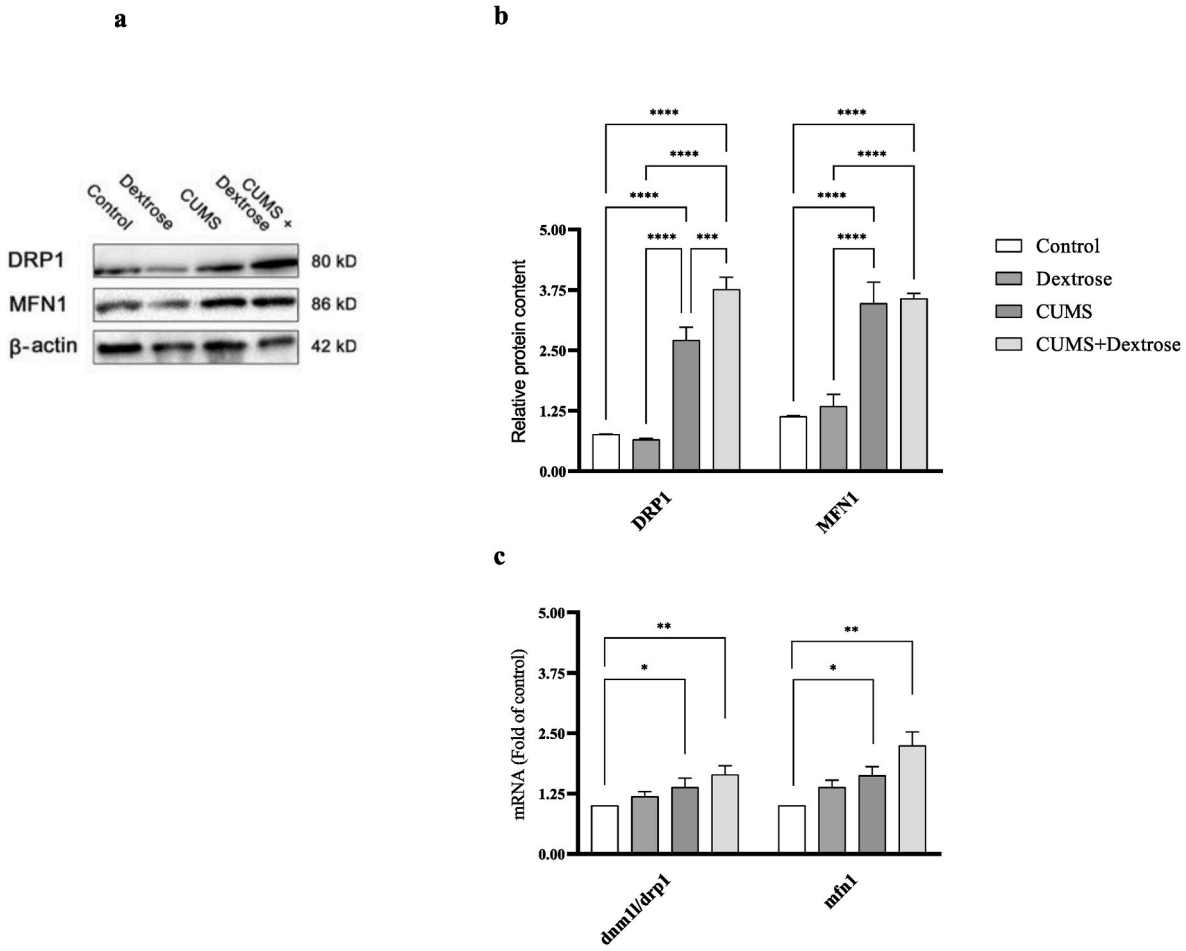

Fig. 8. Effect of CUMS and Dextrose treatment on mitochondrial dynamics: DRP1 and MFN1 representative western blots (a), and relative protein content (b), and relative mRNA quantification of indicated genes: *dnm1l/drp1*, *mfn1* (c). Nonparametric test was used for *dmn1/drp1,* and *mfn1* mRNA content (panel c) statistical analysis. Data presented is the average of three determinations of different homogenates obtained from three pooled zebrafish brains. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) ****, $p < 0.0001$). Each value is represented as mean ± SEM. (n = 3).

groups that parallel the observed increased activity of NRF2, notwithstanding the reduced total NRF2 protein content. Overall, the redox state analysis allowed us to conclude that the oxidative stress condition in CUMS and CUMS + Dextrose treatments induced the activation of a compensative response to counteract the oxidative stress that is more relevant when hyperglycaemia is associated with distress.

Mitochondria are the most important cellular source of ROS production and, at the same time, their target [18]. When the antioxidant defence system does not counterbalance the production of ROS, this can induce mitochondrial dysfunction and damage, triggering or exacerbating CNS disease [60]. Existing evidence for aberrant mitochondrial function and morphology in the brain induced by conditions such as chronic stress and hyperglycaemia highlights ROS as a major contributing factor [61–63].

ROS are also signalling molecules able to affect mechanisms involved in mitochondrial dynamics to face the occurring mitochondrial damage [64]. Increased ROS generation can induce the expression of PGC-1α, a transcriptional co-activator primarily involved in stimulating mitochondrial biogenesis by driving the transcription of other downstream factors, such as NRF1, NRF2/GABP-α and mitochondrial transcription factor A (TFAM) [65]. PGC-1α is the master regulator of mitochondrial quality control and energetic metabolism, and it is also a key factor in maintaining neuronal survival and synaptic transmission [66].

In our experimental model, we found that the increased ROS content and oxidative stress in CUMS and CUMS + Dextrose groups were associated with a significant increase in the content of factors involved in mitochondrial biogenesis, the proteins PGC-1α, NRF1, and NRF2/GABP-α. Our results indicate a predominant role for psychological stress, and since the oxidative stress resulting from mitochondrial dysfunction may play an important role in neurological impairment and cognitive decline [67], we can conclude that the capacity to undergo mitochondrial biogenesis, which is dysregulated in disease states, may play a vital role in determining neuron cell survival, as reported elsewhere [68]. Moreover, the PGC1-α protein also co-activates the NRF2 in inducing the transcription of the antioxidant enzyme genes and, on the other hand NRF2, also activates mitochondrial biogenesis [69]. Accordingly, we found an increased NRF2 activity in both CUMS and CUMS + Dextrose (increased SOD activity and *sod1* mRNA content, GPX activity and tendency to the increase in *gpx* mRNA content, tendency versus the increase in catalase mRNA content) associated with the increase in PGC-1α content. Therefore, this suggests that the processes of NRF2-dependent antioxidant system activation and mitochondrial biogenesis are strictly interconnected highlighting the complexity of the redox regulation of cellular vital processes. Conversely, the respective mRNA content of the mitochondrial biogenesis factors was differently upregulated by the treatments. In CUMS we detected an increased mRNA content of *pgc1-α* and *nrf2/gabpb1,* while in CUMS + Dextrose we detected a significant increase in *nrf1* and *nrf2/gabpb1* mRNA content with a tendency toward augmentation for *pgc1-α*. Furthermore, we did not find any changes in the content of the proteins involved in mitochondrial biogenesis in the whole brain of hyperglycaemic zebrafish, but we found a significant increase in the mRNA content of *nrf1* and a tendency toward the increase

for *nrf2/gabpb1*. Our results disagree with the reduced content of the PGC-1α protein in the brain of streptozotocin-treated mice [70]. Such a difference could depend on differences in treatments or experimental models.

Mitochondrial quality control also involves dynamic mitochondrial morphology changes as a result of the network of fusion and fission processes. Mitochondrial fusion allows the transfer, or sharing, of functional components from healthy mitochondria to supplement compromised ones. The fission separates mitochondria to eliminate the "uncorrectable" parts. Mitochondrial distribution is highly dependent on these two competing yet interrelated processes for neurons [71].

Proteins well conserved in cells during evolution belonging to a family of guanosine triphosphatases (GTPases) are involved in mitochondrial fission and fusion processes: dynamin-related protein 1 (Drp1) for fission, mitofusin1 and 2 (MFN1and MFN2) for outer membrane fusion and Optic atrophy 1 (Opa1) for inner membrane fusion [71].

Our results showed alterations in the levels of DRP1 and MFN1 in CUMS and CUMS + Dextrose treatments, that parallel, at least in part, those of mitochondrial biogenesis. The fusion and fission processes must be coordinated because for proper neuronal cell function [71]. In our experimental model, we found that the increase in DRP1 is higher in CUMS + Dextrose than in CUMS group. Because marked fission was found in conditions of pronounced oxidative stress [72], this result confirms that the association of hyperglycaemia and psychological stress accentuates oxidative stress and the effects on mitochondrial quality control.

6. Conclusion

This research highlighted more detrimental effects for brain redox homeostasis when hyperglycaemia was associated with psychological stress and revealed that the effects did not reflect a simple sum of the alterations induced separately by the treatments. In particular, the hyperglycaemia treatment alone seems not to have induced an oxidative stress condition which, in contrast, resulted more pronounced in CUMS + Dextrose than CUMS treatment. This result suggests the involvement of different molecular mechanisms depending on ROS levels, as supported by the response of the antioxidant enzymatic system, which was amplified in the CUMS + Dextrose group, and mitochondrial quality control mechanisms, that were prompted versus mitochondrial fission to preserve the functionality of the organelles, also in the CUMS + Dextrose group. On the other hand, NRF2, known to be strictly involved in the mechanism adopted to face an oxidative stress condition, also showed an unexpected response to the different treatments, highlighting even more the complexity of its regulation at both translational and post-translational levels. In conclusion, our results underlined the close interdependence between hyperglycaemia and psychological distress: the two phenomena influence each other and cause alterations in oxidative metabolism that can predispose the biological system to the development of functional alterations.

CRediT authorship contribution statement

Rhea Subba: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Gianluca Fasciolo:** Methodology, Investigation, Formal analysis. **Eugenio Geremia:** Methodology, Investigation, Formal analysis. **Maria Teresa Muscari Tomajoli:** Methodology, Investigation, Formal analysis. **Adriana Petito:** Methodology, Investigation, Formal analysis. **Sabrina Carrella:** Writing – review & editing. **Amal Chandra Mondal:** Writing – review & editing, Supervision, Conceptualization. **Gaetana Napolitano:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Paola Venditti:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2024.110101.

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