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Neonatal hyperglycemia induces oxidative stress in the rat brain: the role of pentose phosphate pathway enzymes and NADPH oxidase

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Abstract Recently, the consequences of diabetes on the central nervous system (CNS) have received great attention. However, the mechanisms by which hyperglycemia affects the central nervous system remain poorly understood. In addition, recent studies have shown that hyperglycemia induces oxidative damage in the adult rat brain. In this regard, no study has assessed oxidative stress as a possible mechanism that affects the brain normal function in neonatal hyperglycemic rats. Thus, the present study aimed to investigate whether neonatal hyperglycemia elicits oxidative stress in the brain of neonate rats subjected to a streptozotocin-induced neonatal hyperglycemia model (5-day-old rats). The activities of glucose-6-phosphate-dehydrogenase (G6PD), 6-phosphogluconate-dehydrogenase (6-PGD), NADPH oxidase (Nox), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), the production of superoxide anion, the thiobarbituric acid-reactive substances (TBA-RS), and the protein carbonyl content were measured. Neonatal hyperglycemic rats presented increased activities of G6PD, 6PGD, and Nox, which altogether may be responsible for the enhanced production of superoxide radical anion that was observed. The enhanced antioxidant enzyme activities (SOD, CAT, and GSHPx) that were observed in neonatal hyperglycemic rats, which may be caused by a rebound effect of oxidative stress, were not able to hinder the observed lipid peroxidation (TBA-RS) and protein damage in the brain. Consequently, these results suggest that oxidative stress could represent a mechanism that explains the harmful effects of neonatal hyperglycemia on the CNS.

Keywords Neonatal hyperglycemia · NADPH oxidase · Glucose-6-phosphate-dehydrogenase · Superoxide · Oxidative stress · Brain

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Introduction

In recent years, many clinical pediatric studies have been conducted on neonatal hyperglycemia, a common condition in preterm babies [1–3]. These studies have provided an increased understanding of the genes that are involved in the complete chain of steps that control glucose homeostasis. However, not many studies have addressed the damaging effects of hyperglycemia on the rat brain during CNS development [4].

Recent studies on both diabetic humans and experimentally diabetic adult rats have shown that hyperglycemia induces oxidative stress that may perturb brain function [5]. It is important to emphasize that these investigations studied diabetes type 1 and type 2 in adult or aging populations. To our knowledge, the effect of hyperglycemia on oxidative stress in the developing phase of the CNS (neonatal diabetes) has not yet been reported.

In aerobic metabolism, the production of reactive species is counterbalanced by antioxidant defense systems.

However, the overproduction of reactive species and/or diminished antioxidant defenses may lead to cell injury and death through alterations in the enzymatic systems and membrane transport and through DNA dysfunction due to protein, lipid, and/or DNA oxidative damage [6]. Several hypotheses, such as glycoxidation, the nonenzymatic glycation of proteins, the activation of protein kinase C, a low concentration of antioxidants in tissues, and the impairment of the antioxidant enzyme system, have been developed to explain the elevation of free radicals that is observed in diabetes [7, 8].

The hyperglycemia that is present in diabetes produces alterations in tissue NADPH metabolism that are likely related to alterations in glucose-6-phosphate-dehydrogenase (G6PD), which is a rate-limiting enzyme of the pentose phosphate pathway (PPP) that produces NADPH to be used as the cofactor for other enzymes, such as glutathione reductase and NADPH-dependent oxidases [9]. Moreover, recent studies that used a model of severe hyperglycemia suggest that the Nox-derived superoxide generation in the myocardium is fueled by elevated levels of G6PD-derived NADPH [10]. However, it remains unknown whether these effects of severe hyperglycemia are also present in brain tissue, especially during brain development.

The neurological consequences of diabetes in the central nervous system have been receiving greater attention more recently. However, the mechanisms by which hyperglycemia causes brain damage remain poorly understood. Therefore, to evaluate how the hyperglycemia affects the central nervous system, we investigated various parameters related to oxidative stress in the brain of neonatal rats subjected to a neonatal hyperglycemia model. The following oxidative stress parameters were studied: G6PD and 6-phosphogluconate-dehydrogenase (6PGD) activities to evaluate the main cellular source of NADPH (the oxidative phase of PPP); Nox activity and the content of superoxide anion to evaluate the superoxide anion production; the activities of glutathione peroxidase (GSHPx), catalase (CAT), and superoxide dismutase (SOD) to assess the enzymatic antioxidant defenses; and the thiobarbituric acid-reactive substances (TBA-RS) to assess the lipid peroxidation and protein carbonyl content.

Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The streptozotocin (STZ) solutions were freshly prepared in a mixture containing saline (70 % of final volume) and 20 mM sodium phosphate buffer pH 7.4 (30 % of final volume).

Animals

The NIH Guide for the Care and Use of Laboratory Animals (NIH publication #80–23, revised 1996) was followed in all experiments. Five-day-old Wistar rats, which were bred in the Animal House of Universidade Federal do Rio Grande do Sul, were used according to sample size calculation with 80 % power at the 0.05 level of significance (Minitab®). We used both male and female rats because rats have not yet developed their secondary sexual characteristics by the age at which they were used for the experiments. Except for a brief period of starvation, the rats were maintained with dams, which had free access to water and a 20 % (w/w) protein commercial chow (Germani, Porto Alegre, RS, Brazil), until they were sacrificed. In addition, the rats were maintained in a room with a 12:12 h light/dark cycle (lights on 7:00–19:00 h) and controlled temperature (22 ± 1 °C).

Streptozotocin-induced neonatal diabetes

After 8 h of starvation, the rats developed neonatal diabetes through the intraperitoneal administration of a single injection of STZ (100 mg/kg body weight); the controls received vehicle (saline and buffer) instead of STZ similarly to model utilized by Takada [11]. 10 h after the STZ injection, the rats received an intraperitoneal injection of glucose (2 mg/g body weight) to avoid the development of drug-induced fatal hypoglycemia. 24 h after the STZ administration, we verified the blood glucose (rats with greater than 200 mg/dL glucose were considered hyperglycemic and thus formed part of the diabetic group).

Preparation of tissues

Five days after the STZ injection, the rats were sacrificed by decapitation without anesthesia. The brain was immediately removed and kept on an ice-plate. The olfactory bulb, pons, and medulla were discarded, and the total brain was weighed and maintained chilled until homogenization (1:10, w/v) with 20 mM sodium phosphate buffer pH 7.4 containing 140 mM KCl. The duration of these procedures was at most 5 min. The homogenates were centrifuged at 750 g and 4 °C for 10 min to separate the nuclei and cell debris [12, 13]. The pellet was discarded, and the supernatant was immediately separated and used for the measurements.

Glucose-6-phosphate-dehydrogenase (G6PD) and 6-phosphogluconate-dehydrogenase (6-PGD) assays

The activities of G6PD and 6PGD were measured through the monitoring of the production of the coenzyme NADPH.

To obtain accurate enzyme activities, the 6PGD activity and total dehydrogenase activity (G6PD + 6PGD) were measured separately, and the difference between these two measures was related to the G6PD activity. To obtain the total dehydrogenase activity, the substrates for both dehydrogenase enzymes were added to a cuvette. The reaction mixture contained 100 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$, 1 mM 6-phosphogluconate (6PGD substrate), 1 mM glucose-6-phosphate (G6PD substrate), and the sample. In another cuvette, the same reaction mixture without glucose-6-phosphate was used to obtain the activity of 6PGD alone. The G6PD activity was calculated by subtracting the activity of 6PGD from the total dehydrogenase activity (G6PD = total − 6PGD). The reactions were started through the addition of 1 mM $NADP^+$ and monitored in a spectrophotometer at 340 nm. One enzyme unit was defined as 1 μmol of substrate transformed per min. The specific activity is represented as the number of units per mg of protein [14, 15].

NADPH oxidase (Nox) assay

The NADPH oxidase activity was determined by measuring the chemiluminescence intensity of lucigenin, which is induced by superoxide production after the addition of NADPH to a luminometer counter [16]. Briefly, 273 μL of air-equilibrated Krebs-HEPES buffer (99 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, 1.9 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.1 mM glucose, and 20 mM HEPES-Na pH 7.44) was added to a plate and maintained at 37 °C. Then, 6 μL of 20 μM lucigenin was added, and the background level of chemiluminescence was counted for 2 min. The sample (15 μL) and NADPH (6 μL) were then added and counted for 5 min to determine the kinetic activity of NADPH oxidase, which is based on the increasing lucigenin chemiluminescence due to superoxide production. The NADPH oxidase activity was represented as the amount of RLU per min per mg of tissue.

Superoxide radical anion ($O_2^{\cdot-}$) content

The superoxide content was determined by measuring the chemiluminescence intensity of lucigenin [16]. Briefly, 194 μL of air-equilibrated Krebs-HEPES buffer (99 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, 1.9 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.1 mM glucose, and 20 mM HEPES-Na pH 7.44) was added to a plate and maintained at 37 °C. Then, 6 μL of 20 μM lucigenin was added, and the background level of chemiluminescence was counted for 5 min. The sample (100 μL) was then added and counted for 10 min to determine the content of superoxide anion in the tissue. The background was then subtracted from the values obtained from the tissue fraction-added incubation. The chemiluminescence data are represented as the amount of RLU per min per mg of tissue.

Superoxide dismutase (SOD) assay

The assay of the SOD activity, which was described by Marklund [17], is based on the autoxidative capacity of pyrogallol. Autoxidation is a process that is highly dependent on superoxide, which is the substrate for SOD. The autoxidation of pyrogallol is inhibited in the presence of SOD. The activity of SOD can then be indirectly spectrophotometrically assayed at 420 nm using a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). A calibration curve was generated using purified SOD as the standard to calculate the activity of SOD that is present in the samples. The results are represented as the amount of SOD units per mg of protein.

Glutathione peroxidase (GSHPx) assay

The GSHPx assay was performed according to the method described by Wendel [18] using *tert*-butyl hydroperoxide as the substrate. The NADPH disappearance was monitored at 340 nm using a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide, and 0.1 mM NADPH. One GSHPx unit was defined as 1 μmol of NADPH consumed per min. The specific activity is represented as the number of GSHPx units per mg of protein.

Catalase (CAT) assay

The CAT activity was assayed through the method developed by Aebi [19] using a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1 % Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per min. The specific activity is represented as the number of CAT units per mg of protein.

Thiobarbituric acid-reactive substances (TBA-RS)

The amount of TBA-RS was measured according to the method developed by Ohkawa [20]. Briefly, the following were added to Eppendorf tubes, in order: 150 μL of tissue homogenate, 40 μL of 8.1 % SDS, 280 μL of 20 % acetic acid in aqueous solution (v/v) pH 3.5, and 280 μL of 0.8 %

thiobarbituric acid. After the mixture was vortexed, the reaction was performed in a boiling water bath for 1 h. The mixture was allowed to cool on water for 5 min and was then centrifuged at 750 g for 10 min. The number of resulting pink-stained TBA-RS was determined spectrophotometrically at 535 nm in a Beckman DU®640 Spectrophotometer. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as the standard, which was subjected to the same treatment as the samples. The amount of TBA-RS is represented as nmol per mg protein.

Protein carbonyl content

Protein carbonyl content was assayed by a method based on the reaction of carbonyls with dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm [21]. Briefly, 200 µL of sample was added to 400 µL 10 mM DNPH in 2 M HCl, or to 2 M HCl (blank). This mixture was kept in dark room for 1 h and vortexed each 15 min. After that, 500 µL of 20 % trichloroacetic acid (TCA) was added to each tube. The mixture was vortexed and centrifuged at $20{,}000 \times g$ for 3 min. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed, and centrifuged at $20{,}000 \times g$ for 3 min to remove the free DNPH. The supernatant was discarded, and the pellet was resuspended in 600 µL of 6 M guanidine (prepared in a 20 mM potassium phosphate pH 2.3), vortexed, and incubated at 60 °C for 15 min. After that, it was centrifuged at $20{,}000 \times g$ for 3 min, and the supernatant was used to measure absorbance in a spectrophotometer at 370 nm. The molar extinction coefficient of 22,000 for DNPH was used to calculate the concentration of carbonyls content, and the results were reported as nmol/mg protein.

Protein determination

The protein concentration in the brain homogenates was determined using bovine serum albumin as the standard [22].

Statistical analysis

The statistical analysis was performed using Student's t test. All of the analyses were performed using the GraphPad Prism 5 software in a PC-compatible computer. A value of $p < 0.05$ was considered statistically significant.

Results

There was a significant increase in the glycemia of the neonatal diabetes group (222 mg/dL) compared to the control (121 mg/dL) [$t(38) = 3.42$; $p < 0.01$]. Furthermore, to prove that the model is efficient, we found that the diabetic animals (received STZ) presented less insulin than control animals, approximately five times lower [$t(6) = 4{,}631$; $p < 0.01$] (data not shown). In the present study, we also investigated the effects of neonatal hyperglycemia on several oxidative stress parameters in the brains of rats.

We first investigated the effect of STZ-induced neonatal hyperglycemia on the activities of G6PD and 6PGD, which are the two enzymes that produce NADPH in the oxidative phase of the PPP (Fig. 1). As shown in Fig. 1a and b, we observed that the G6PD [$t(12) = 3.97$; $p < 0.01$] and 6PGD [$t(12) = 3.5$; $p < 0.01$] activities were significantly increased in hyperglycemic rats compared to controls.

We then evaluated the effect of neonatal hyperglycemia on the superoxide anion production and on the activity of Nox (Fig. 2). Neonatal hyperglycemia was able to significantly increase the superoxide anion production [$t(14) = 3.073$; $p < 0.01$] and the Nox activity [$t(9) = 2.565$; $p < 0.05$], as shown in Fig. 2a and b, respectively.

We also studied the effect of neonatal hyperglycemia on the brain enzymatic antioxidant defenses by assessing the activities of the antioxidant enzymes SOD, CAT, and GSHPx. As shown in Fig. 3a, neonatal hyperglycemia significantly enhanced the activity of SOD [$t(13) = 6.38$; $p < 0.001$]. In addition, the CAT [$t(11) = 2.27$; $p < 0.05$] and GSHPx [$t(13) = 3.97$; $p < 0.01$] activities in the total brains of rats were also enhanced by neonatal hyperglycemia (Fig. 3b, c, respectively). These results clearly show an increment in the enzymatic antioxidant defenses in the total brains of rats that developed STZ-induced neonatal hyperglycemia.

We evaluated the effect of neonatal hyperglycemia on the lipid peroxidation parameter TBA-RS. Figure 4a depicts that neonatal hyperglycemia markedly enhanced the TBA-RS levels in the total brain homogenates [$t(15) = 3.47$, $p < 0.01$]. These results indicate that neonatal hyperglycemia promotes lipid peroxidation in brain tissue homogenates.

We also measured the effect of neonatal hyperglycemia on protein carbonyl content as a protein damage parameter. Figure 4b depicts that neonatal hyperglycemia markedly enhanced the protein carbonyl content in the total brain homogenates [$t(18) = 4.44$, $p < 0.001$]. These results indicate that neonatal hyperglycemia promotes protein damage in brain tissue homogenates.

Discussion

The consequences of hyperglycemia on the central nervous system have been the subject of increasing interest. Some

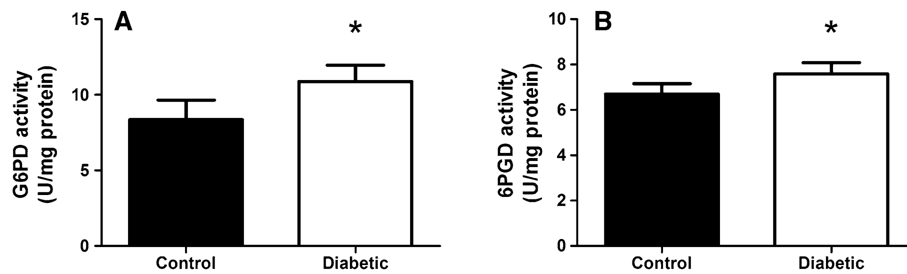

Fig. 1 Effect of neonatal hyperglycemia on the activities of glucose-6-phosphate-dehydrogenase (G6PD) (**a**) and 6-phosphogluconate-dehydrogenase (6PGD) (**b**) in the rat brain. Results are the mean ± SD ($n = 6$–8) of independent experiments performed in duplicate. *$p < 0.05$ and **$p < 0.01$ compared to control (Student's t test for unpaired samples)

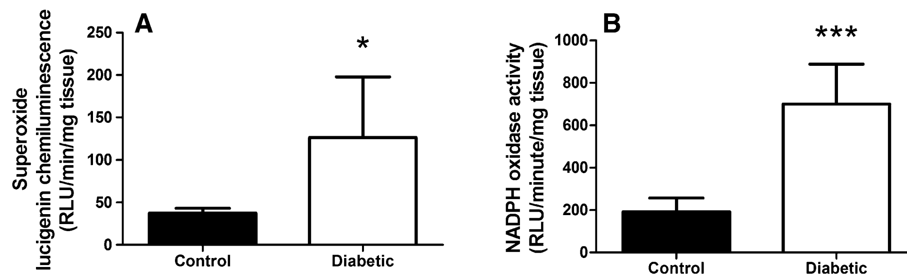

Fig. 2 Effect of neonatal hyperglycemia on superoxide anion content (**a**) and NADPH oxidase (Nox) activity (**b**) in the rat brain. Results are the mean ± SD ($n = 6$–10) of independent experiments performed in duplicate. *$p < 0.05$ and **$p < 0.01$ compared to control (Student's t test for unpaired samples)

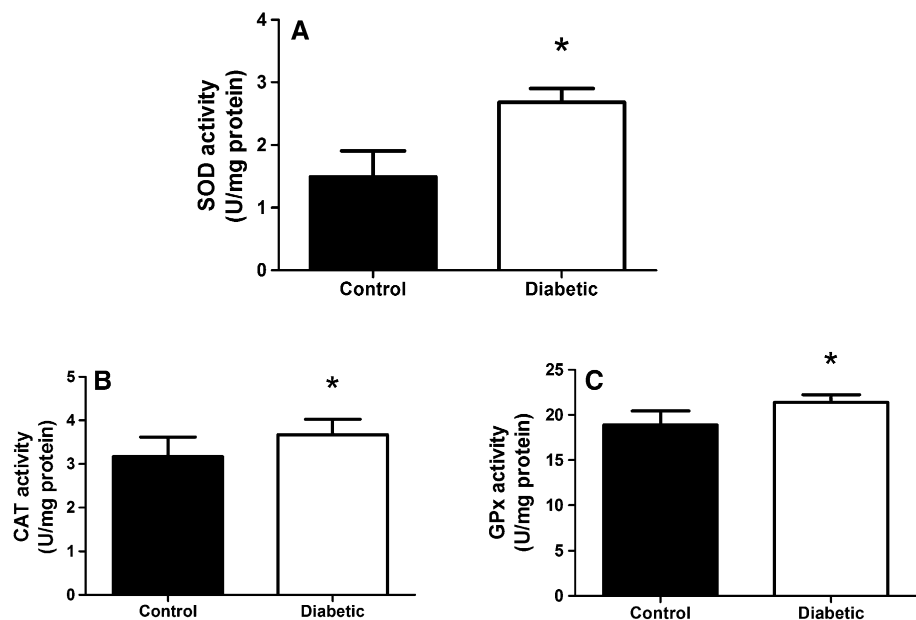

Fig. 3 Effect of neonatal hyperglycemia on the activities of superoxide dismutase (SOD) (**a**), catalase (CAT) (**b**), and glutathione peroxidase (GSHPx) (**c**) in the rat brain. Results are the mean ± SD ($n = 6$–10) of independent experiments performed in duplicate. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ compared to control (Student's t test for unpaired samples)

studies have shown that hyperglycemia produces molecular, cellular, and morphological changes in the central nervous system of adult rats [23]. Although the mechanisms by which neonatal hyperglycemia affects the central nervous system remain not fully understood, several lines of evidence indicate that the oxidative damage induced by hyperglycemia may contribute to nervous system damage [24, 25]. It is important to note that the literature on neonatal hyperglycemia provides data on the clinical aspects of this disease, but not on the effects and mechanisms by which it may affect the neonatal rat brain.

Under normal physiological conditions, there is a critical balance in the generation of free radicals and the development of the antioxidant defense system that is used by organisms to deactivate these reactive species and to protect against free radical toxicity [26]. An impairment in

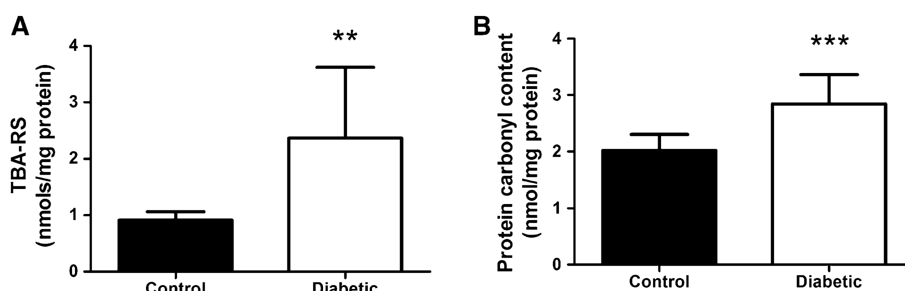

Fig. 4 Effect of neonatal hyperglycemia on thiobarbituric acid-reactive substances (TBA-RS) (**a**) and protein carbonyl content (**b**) in the rat brain. Results are the mean ± SD ($n = 6$–9) of independent experiments performed in duplicate. $*p < 0.05$; $**p < 0.01$ compared to control (Student's t test for unpaired samples)

the oxidant/antioxidant equilibrium creates a condition known as oxidative stress, in which free radicals can cause oxidative injury by attacking macromolecules, such as lipids, carbohydrates, proteins, and nucleic acids [6]. Oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases and has been associated with the pathogenesis of diabetes and its complications, likely due to hyperglycemia [27, 28]. The hyperglycemia that is present in diabetes is usually accompanied by an increased production of free radicals [7, 29, 30] and/or the impairment of antioxidant defenses [31, 32]. Hyperglycemia, which is a factor that is shared by all forms of diabetes, is a major contributor to oxidative stress through either the direct generation of reactive oxygen species (ROS) or the alteration of the redox balance [33]. Most investigations on the mechanisms underlying hyperglycemia-induced oxidative stress studied vascular tissue and focused on the five major mechanisms: the increased flux of sugars through the polyol pathway, the increased intracellular formation of advanced glycation end products (AGEs), the increased expression of the receptor for AGEs, the activation of protein kinase C (PKC) isoforms, and the overactivity of the hexosamine pathway. Compelling evidence has led to the hypothesis that all of these mechanisms are activated by a single upstream event: an increased production of superoxides in vascular tissue [34]. In addition, recent studies have demonstrated that the overexpression of G6PD increases NADPH production, which in turn enhances the superoxide anion production by Nox in the heart and vasculature [10, 35, 36].

Therefore, based on the present evidence, we investigated the effects of STZ-induced neonatal hyperglycemia on several oxidative stress parameters in the brains of developing rats. To identify a possible mechanism by which oxidative stress plays a role in neonatal hyperglycemia-induced neurotoxicity, we also evaluated whether NADPH oxidase is a source of superoxide anions and whether G6PD and 6PGD-derived NADPH may be involved in the increased generation of superoxide anions in neonatal diabetes.

The overall rate of brain glucose utilization is regulated in an integrated, complex manner by various regulatory metabolites at many steps. On the other hand, the glucose 6-phosphate flux through PPP pathway is mediated by $NADP^+$ availability, which in turn is determined by the consumption of NADPH due to, mainly, the regeneration of GSH from GSSG as the cell manages oxidative stress [37].

Thus, we first measured the effect of neonatal hyperglycemia on the G6PD and 6PGD activity because these are the fundamental enzymes of the PPP that produces NADPH, which is required for many reduction systems and for NADPH oxidase activity. We observed that neonatal hyperglycemia was able to significantly increase the G6PD and 6PGD activities in rat brains, which is in accordance with the results presented by Ulusu [38], who found increased G6PD activity in the brains of adult rats subjected to hyperglycemic conditions. Also, a recent study indicated that chronically elevated glucose levels enhance PPP activity, and that acute increases in glucose levels stimulate both glucose utilization and PPP activity to minimize ROS production and sustain levels of GSH in primary astroglial cultures from 2-day-old rats. These actions of astroglia indicate a protective role against oxidative stress in the brain under the hyperglycemic conditions associated with diabetes mellitus [39].

We then confirmed that neonatal hyperglycemia was able to increase both superoxide anion levels and NADPH oxidase activity. These findings are in accordance with studies that have shown increased superoxide anion production in the brains of adult rats with hyperglycemia [40–42]. NADPH oxidases are expressed by a wide variety of cells (e.g., Nox3 by brain) generating superoxide anion and hydrogen peroxide and are considered an important source of superoxide anion besides mitochondrial electron transport chain [43]. It is now known that high levels of glucose are associated with enhanced ROS into cells, mainly

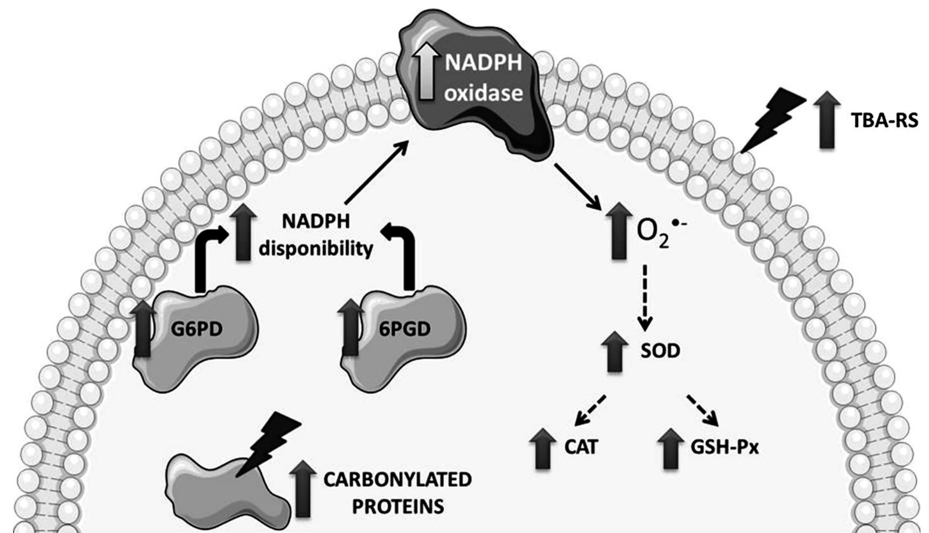

Fig. 5 Overview of the effects of neonatal hyperglycemia on glucose-6-phosphate-dehydrogenase (G6PD), 6-phosphogluconate-dehydrogenase (6PGD), superoxide anion ($O_2^{\cdot -}$), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), lipid peroxidation, and protein carbonylation in rat brain. The figure was produced using Servier Medical Art (www.servier.com)

generated by mitochondria and NADPH oxidases. So, hyperglycemia can stimulate the overproduction of superoxide and hydrogen peroxide, themselves precursors of other reactive species, which may exhaust antioxidant systems, damage many biomolecules, and increase lipid peroxidation [44].

Enzymatic antioxidants, such as SOD and CAT, play an important role in the prevention of cells from exposure to oxidative damage [45]. SOD is an enzymatic antioxidant that scavenges superoxide radicals by catalyzing the conversion of this free radical into hydrogen peroxide and molecular oxygen [46]. CAT, which is another enzymatic antioxidant, catalyzes the reduction of hydrogen peroxide and protects tissues against further production of reactive hydroxyl radicals through a Fenton reaction. GPx is considered biologically essential in the reduction of hydrogen peroxide and other peroxides. The analysis of the major enzymatic antioxidant defenses showed that neonatal hyperglycemia significantly enhanced the activities of SOD, CAT, and GSHPx in the brains of rats. The antioxidant enzyme activities have been shown to both increase and decrease in adult rat brains with experimental diabetes depending on both the duration and the severity of the disease [47, 48]. In this study, we demonstrate an increase of superoxide radical production in the brain of rats subjected to neonate hyperglycemia. Superoxide is the first oxygen free radical formed by the mitochondria, which is then converted to other more reactive species that can damage cells, as seen in part in this work by the increase of lipid peroxidation (TBARS) [49]. Under hyperglycemia, more glucose-derived pyruvate is available to be oxidized in the TCA cycle, increasing the flux of electron donors (NADH and $FADH_2$) into the electron transport chain. This effect increases the voltage gradient across the mitochondrial membrane reaching a critical threshold that blocks electron transfer inside complex III, causing the electrons to back up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating more superoxide when compared to cell under normal glycemia. Superoxide radical is degraded by SOD to hydrogen peroxide, which is eventually converted to H_2O and O_2 by other enzymes (CAT and GSHPx) [34]. So, we can speculate that the increased activities of SOD, CAT, and GSHPx may reflect a higher synthesis of these enzymes due to a rebound effect against oxidative stress in an effort to counteract the overproduction of superoxide anion.

Neonatal hyperglycemia significantly enhanced protein damage in rat brain, which was assessed by protein carbonyl content. Interestingly, oxidative stress has been related to dicarbonyl-induced glycation of amino acids of susceptible proteins which may be involved in the pathophysiological mechanisms of both diabetes and neurodegenerative diseases [50]. High glucose can stimulate the production tissue dicarbonyl levels, mainly the high-reactive methylglyoxal (MGO). In addition, the degradation of MGO by glyoxalase system is dependent of GSH [51].

We then investigated the effect of neonatal hyperglycemia on lipid peroxidation, which was assessed by the classic parameter TBA-RS. We observed that hyperglycemia significantly enhanced the TBA-RS levels in the brains of neonate rats, which indicates that neonatal hyperglycemia promotes lipid peroxidation. The TBA-RS levels reflect the amount of malondialdehyde, which is the end product of lipid peroxidation [6]. Therefore, the enhancement of this parameter due to neonatal hyperglycemia suggests an induction of lipid oxidative damage due to oxidative stress. This result is in accordance with many studies that have shown hyperglycemia-induced lipid peroxidation in adult rat brains [38, 47, 48, 52–54]. It should be noted that some reactive species, such as

hydrogen peroxide and superoxide anion, are not directly able to trigger lipid peroxidation, whereas hydroxyl radicals and peroxynitrite can directly initiate oxidative damage to lipids [6]. Therefore, the enhanced superoxide anion production by neonatal hyperglycemia can led to the production of other reactive species, which can directly initiate lipid peroxidation.

As outlined in Fig. 5, the results of the present study suggest that neonatal hyperglycemia increases superoxide anion levels in the brain by enhancing the activities of G6PD, 6PGD, and NADPH oxidase. Lipid peroxidation and protein carbonyl content were also increased along with the observed stimulation of the activities of the antioxidant enzymes (SOD, CAT, and GSHPx). These effects may point to possible therapeutic targets that can be further studied for the treatment of this pathological condition. It should be emphasized that the central nervous system is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, particularly polyunsaturated fatty acids, and its low activity of antioxidant defenses [27]. Because oxidative stress can be elicited by an imbalance between free radical production and antioxidant defenses and because neonatal hyperglycemia induces an increase in reactive species (superoxide anion), lipid and protein oxidative damage, we postulate that oxidative stress may contribute, along with other known mechanisms, to the possible neurological damage that is induced by hyperglycemia in the crucial period of brain development.

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Conflicts of interest The authors declare that they have no conflicts of interest.

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