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Effects of insulin and clonazepam on DNA damage in diabetic rats submitted to the forced swimming test

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ABSTRACT

Diabetes mellitus (DM) is a chronic hyperglycemic state. DM may be associated with moderate cognitive deficits and neurophysiologic/structural changes in the brain (diabetic encephalopathy). Psychiatric manifestations seem to accompany this encephalopathy, since the prevalence of depression in diabetic patients is much higher than in the general population, and clonazepam is being used to treat this complication. The excessive production of oxygen free radicals that may occur in diabetes induces a variety of lesions in macromolecules, including DNA. In this work, we analyzed DNA damage in leukocytes from streptozotocin-induced diabetic rats submitted to the forced swimming test. The DNA damage index was significantly elevated (DI = 61.00 ± 4.95) in the diabetic group compared to the control group (34.00 ± 1.26). Significant reductions of the damage index were observed in diabetic animals treated with insulin (45.00 ± 1.82), clonazepam (52.00 ± 1.22), or both agents (39.00 ± 5.83, not significantly different from control levels). Insulin plus clonazepam can protect against DNA damage in stressed diabetic rats.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by a hyperglycemic chronic state that can lead to complications affecting the retina, kidney, muscle, blood vessels, and also the central nervous system (CNS). DM is regarded as one of the major metabolic diseases of the 21st C. [1,2]. Cognitive dysfunction is developed by some diabetic individuals [3]. A wealth of studies have described neuropsychological and neurobehavioral changes in both type 1 and type 2 diabetic subjects, suggesting that diabetic encephalopathy should be recognized as a complication of diabetes [4,5]. Psychiatric manifestations may accompany this encephalopathy, since the prevalence of depression in diabetic patients is much higher than in the general population [6–9].

Diabetic encephalopathy can be modeled in experimental animals; diabetic mice and rats exhibit depressive-like behavior when submitted to the forced swimming test (FST) [10–12]. Some studies have demonstrated that insulin administration can prevent neuronal damage in the cortex of streptozotocin (STZ)-induced diabetic rats and also produce behavioral changes [11,13]. Insulin also affects synaptosomal γ-aminobutyric acid (GABA) and glutamate transport under oxidative stress conditions [14] and clonazepam (CNZ), a positive $GABA_A$ receptor modulator, shows an antidepressant effect in these animals [10,15].

Oxidative stress may play a role in the development of diabetic complications [16], since hyperglycemia generates abnormally high levels of free radicals via autoxidation of glucose and protein glycation [17]. These free radicals induce a variety of lesions in DNA, including oxidized bases, abasic sites, strand breaks, and formation of DNA–protein cross-links. Hydroxyl radical, produced by the Fenton reaction in the presence of transition metal ions, is responsible for this DNA damage [18].

Using the comet assay, we have investigated DNA damage in peripheral whole blood leukocytes from streptozotocin-induced diabetic rats submitted to the FST, and we have evaluated the effects of acute treatment with insulin and/or clonazepam upon this process.

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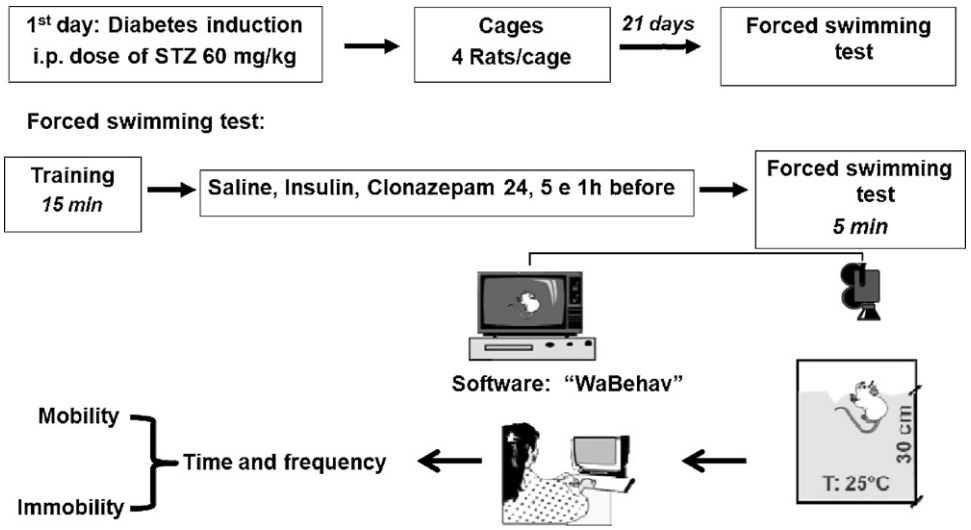

Fig. 1. Time-line chart of the experiment.

2. Materials and methods

2.1. Animals

Male Wistar rats (250 ± 50 g) were obtained from the Animal House of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). The animals were housed in groups of four per polypropylene cage. Food and water were available *ad libitum*, except where otherwise stated, and the animals were maintained in a temperature-controlled room (22 ± 2 °C) under a light–dark cycle (7:00 a.m.–7:00 p.m.). The animals were divided into five groups: control (nondiabetic); diabetic (STZ); diabetic plus insulin (STZ–INS); diabetic plus clonazepam (STZ–CNZ); and diabetic plus insulin and clonazepam (STZ–INS–CNZ). All *in vivo* experiments followed the guidelines of the International Council for Laboratory Animal Science (ICLAS) and were approved by the Ethical Committee for Animal Experimentation of UFCSPA (08404). All efforts were made to minimize animal suffering and to use only the number of animals necessary to generate reliable data.

2.2. Drugs

Insulin (dose, 4 IU/mL) was administered intraperitoneally (i.p.) (Humulin®, Lilly, USA). CNZ (0.25 mg/mL; Rivotril®, Roche, Brazil) was prepared in saline with Tween 0.05% (v/v) and streptozotocin (60 mg/mL; Sigma, St. Louis, MO, USA) was prepared in citrate buffer (pH 4.3). All solutions were prepared immediately before i.p. administration.

2.3. Diabetes induction

Diabetes was induced by a single i.p. dose of STZ, 60 mg/kg, as already described [10]. Increased blood glucose levels (≥250 mg/dL) of STZ-rats (blood collected from tail) were confirmed with a glucometer (AccuChek Aviva®, Roche, Germany) after 72 h. Nondiabetic control rats received i.p. injections of saline (1 mL/kg) and were also submitted to blood glucose measurement.

2.4. Forced swimming test (FST)

The design of the experiments is summarized in Fig. 1. After 21 days of diabetes induction, animals were submitted to the FST [10,19]. On the first day of the experiment, 24 h before the FST, the animals were placed in the aquarium for 15 min (22 cm × 22 cm × 35 cm) with water of height 27 cm (temperature, 24–26 °C). Soon after, the rats were dried with towels and the first drug dose was administered (insulin (4 IU/kg i.p.), clonazepam (0.25 mg/kg i.p.), insulin + clonazepam or saline (1 mL/kg i.p.)). 5 h and 1 h before being submitted to the FST, the animals were again dosed with the indicated treatment. The FST session was recorded on videotape for subsequent analysis.

2.5. Blood sample

Animals were sacrificed by decapitation 30 min after the FST. Whole blood was collected aseptically in heparinized vials and stored at 4 °C until analysis.

2.6. Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described by Singh et al. [20] in accordance with general guidelines for use of the assay [21,22]. Isolated rat leukocytes were suspended in agarose and spread onto a glass microscope slide pre-coated with agarose. Agarose was allowed to set at 4 °C for 5 min. Slides were incubated in ice-cold lysis solution [12.35% NaCl (2.5 M), 3.15% EDTA (100 mM), 0.10% TRIS (10 mM), 0.68% NaOH (30 mM), 0.84% sodium sarcosinate, 82.88% distilled water] to remove cell proteins, leaving DNA as "nucleoids". After the lysis procedure, slides were placed on a horizontal electrophoresis unit and covered with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali-labile-sites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). The cells were then neutralized, washed in double-distilled water, and stained according to a silver-staining protocol [23]. After drying at room temperature overnight, gels were analyzed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration) according to tail intensity. Therefore, the damage index (DI) for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analyzed under blind conditions by at least two different individuals.

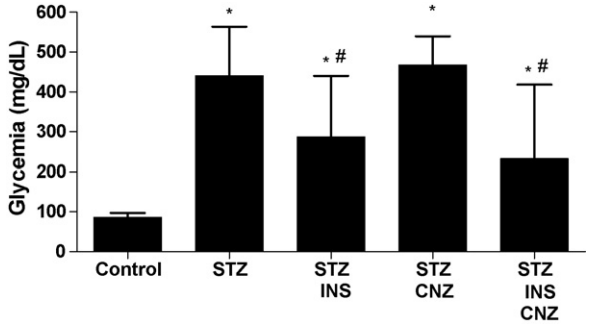

Fig. 2. Glycemia from streptozotocin-induced diabetic rats not treated (STZ) or treated with insulin (STZ–INS), clonazepam (STZ–CNZ), or insulin + clonazepam (STZ–INS–CNZ) and submitted to the forced swimming test (n = 12–13), and controls (n = 8). Data represent mean ± S.D. $^{*}p < 0.05$ compared to the control; $^{\#}p < 0.05$ compared to the diabetic group (ANOVA followed by the Duncan test).

2.7. Statistical analyses

Blood glucose measurement and comet assay data were expressed as mean ± standard deviation and analyzed by one-way analysis of variance (ANOVA), followed by the Duncan multiple range test when the F value was significant. A p value <0.05 was considered significant. The Pearson correlation test was used to evaluate the correlation between the variables. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

Table 1 shows the DI values and the number of cells found in each damage class for each of the five groups of animals. Fig. 2