OXIDATIVE STRESS IN STREPTOZOTOCIN-INDUCED EXPERIMENTAL DIABETES IN RATS IS ASSOCIATED WITH CHANGES OF ANTIOXIDANT STATUS OF HEART TISSUE.

Résumé

L’objectif de notre travail est d’étudier chez le rat mâle Wistar les effets d’un stress oxydatif chronique diabétogène, durant une période de douze semaines sur l’évolution de certains marqueurs de présence de radicaux libres au niveau du tissu cardiaco. Nos résultats montrent une augmentation très significative de l’activité de la superoxide dismutase, glutathion peroxidase et de la glucose-6-phosphate déshydrogénase pendant toute la durée de l’expérimentation. L’activité de la catalase marqueur de la formation du peroxide d’hydrogène, augmente initialement après la deuxième et la troisième semaine et à la douzième semaine de l’expérimentation. La teneur tissulaire en acide ascorbique, un des composés piègeur de radicaux libres, diminue significativement la 3ème et la 6ème semaine puis augmente à la 12ème semaine. Les niveaux des LPO, indicateurs de la peroxydation des lipides augmentent très significativement en fin d’expérimentation. Nos résultats suggèrent que l’hyperglycémie entraîne une augmentation des marqueurs de la présence de radicaux libres au niveau du tissu cardiaco et les mécanismes de défense de cet organe sont modérément efficaces.

Mots clés: Streptozotocine; Antioxydant; hyperglycémie; Diabète; cœur; Rat.

Abstract

The present study was designed to evaluate the oxidative stress-related parameters in streptozotocin-induced diabetes in rats. After 2, 3, 6 and 12 weeks of hyperglycaemia the enzymatic parameters were measured in heart tissue of diabetic and control groups. Superoxide dismutase, glutathione peroxidase and the glucose-6-phosphate dehydrogenase activities significantly increased at all time intervals. Catalase activity increased initially (after 2 and 3 weeks), decreased after 6 weeks and increased again at the 12th week of the experiment. Glutathione reductase activity increased initially (at 2nd week), decreased below control level after 3 weeks, and then increased again. Ascorbic acid concentration decreased after 3 and 6 weeks, and increased at the 6th and 12th weeks. The level of lipid peroxidation products was reduced after 2, 3 and 6 weeks of the experiment. After 12 weeks it was significantly elevated. These data suggest that chronic hyperglycaemia induces oxidative stress in the heart but the defense mechanisms in the heart tissue are fairly efficacious against oxidative injury.

Keywords: Streptozotocin; Antioxidant; Hyperglycaemia; Diabetes; Heart; Rat.

ملخص

الهدف من هذا العمل هو دراسة تأثير توتر تاكسيدي الناتج عن ارتفاع مرن في نسبة تركيز السكر في الدم خلال فترة تصل إلى أسبوعين على انخفاض بعض المؤشرات الأنزيمية قدر أن يوجد الجذور الحرة على مستوى سهولة القلب. النتائج المحصل عليها تبين ارتفاع مض ضوي للنشاط G6PD و بين سكرين Dion و Catalase و تحكم بين H2O2، الكثير في بداية التجربة (اسبوع أول وثاني) و (رابع وثاني) C-SOD-G6PD و تتغذى في النشاط G6PD التي تبين ارتفاع مض ضوي في قلب القلب الذي يعتبر من أحد المكونات المحالة للجذور الحرة. الانخفاض معنوي خلال الأسبوع 3 و 6 و تتم بعد ذلك في الأسبوع 12 ل مستوى النتائج. ي koty الصغير نتائج التكاثر لمستوى LPO الذي يعتبر كمتر للأنسجة الدهنية الإبداعية و يوفر معيونا في آخر التجربة. من نتائج التجربة، فإن سهولة التكاثر المحصل عليها تستطيع أن تكون في نسبة السكر في الدم تؤدي إلى ارتفاع المؤثرات الأنزيمية قدر أن يوجد الجذور الحرة في النتائج القلبية (ضغط التكاثر) على مستوى هذا العضو و البداعة. النتائج النتائج التأسيدي على مستوى هذا السباع فعالة نسبيا. الكلمات المفتاحية: ستراتوزوتوسين، مضاد التكاثر، داء السكري، القلب، فارا.
On the other hand, oxidative stress is involved in the origin of type1 diabetes, especially via apoptosis of pancreatic β-cells, as well as insulin resistance in type 2 diabetes [3].

There is also evidence that elevation in glucose concentration may depress natural antioxidant defense agents such as vitamin C or glutathione [8, 22]. Recent experimental findings suggest that overproduction of reactive oxygen and nitrogen species, lowered antioxidant defense and alterations of enzymatic pathways in humans with poorly controlled diabetes mellitus can contribute to endothelial, vascular and neurovascular dysfunction [9,10,25].

Over the past decade, there has been substantial interest in oxidative stress and its potential role in diabetogenesis, development of diabetic complications, atherosclerosis and associated cardiovascular disease. Cardiovascular disease is the leading cause of mortality in patients with diabetes. Myocardial infarction and stroke constitute the cause of death in as many as 80% of subjects with type 2 diabetes [6].

Several studies in human and animal models have shown alterations in the antioxidant status in diabetes. Especially, disturbances in the defense system in various tissues from animals with experimental diabetes are reported. So far, a number of experiments have been performed to evaluate changes in the antioxidative system of the diabetic heart [13,2,4]. The activity of superoxide dismutase (SOD) increased in diabetic myocardium during seven weeks of the disease [11,27,23]. Matkovics et al. [18] reported that the SOD activity in the heart was mildly elevated 48 h after alloxan treatment. The activity of catalase (CAT) was markedly elevated in diabetic myocardium during 32weeks [23] and after 48 h of diabetes [18]. Similar results were obtained by Tatsuki et al. [24] and by Kakkar et al. [11]. On the other hand, Yadav et al. [28] reported that CAT activity in the diabetic heart was not affected. Total glutathione (GSH) level was slightly increased in the heart tissue at the 15th week of diabetes [17]. Kakkar et al. [11] stated that glutathione peroxidase (GSH-Px) activity in the heart increased significantly during 6 weeks of the experiment. Parninandi et al.[21] reported an increase in the level of GSH and GSH-Px activity. On the other hand, Yadav et al. [28] reported that GSH content and glutathione reductase (GSSG-R) activity in the heart tissue were markedly lowered. In the study of Doi et al.[4] the GSH level was also significantly reduced. Sun et al.[24] stated that ascorbic acid concentration of diabetic hearts decreased significantly after 8 weeks of the experiment. Parninandi et al.[21] did not observe an intensification of lipid peroxidation (LPO) in the diabetic heart. In other experiment the level of LPO products significantly increased 2 weeks after the initiation of diabetes, after 7 weeks it returned to the level of the control [26]. An increase of lipid peroxidation was also observed in the cardiac muscle tissue during 32 weeks of diabetes [23].

We decided to examine the long-term effect of the diabetogenic streptozotocin by determining the parameters of the antioxidative status in diabetic heart at various stages of development of the disease (2, 3, 6 and 12 weeks). We examined the enzymatic antioxidants SOD, CAT, GSH-Px, GSSG-R, glucose-6 phosphate déshydrogénase G6PD and non-enzymatic parameter vitamin C. Lipid peroxidation was also monitored at each stage of the experiment.

MATERIALS AND METHODS

Experimental animals

The institutions guide lines for the care and use of laboratory animals was observed. The animals used were adult male Wistar rats weighing 250-300g. The animals were obtained from Pasteur Institute of Algeria. They were group housed in polyacrylic cages with not more than 4 animals per cage and maintained under standard laboratory conditions with natural dark and light cycle. The rats were fed standard diet and were given water ad libitum. Diabetes was induced by a single intraperitoneally injection of streptozotocin (50 mg/kg, Sigma Chemical, St. Louis, USA) in citrate buffer (0.05 mol/L, pH 4.5). Control animals were injected with same volume of citrate buffer. Seven days after streptozotocin administration serum glucose concentration was measured by glucose oxidase method [34]. All streptozotocin-treated animals in which glucose concentration in the serum was lower than 11mmol/l were excluded from the experiment. Glucose concentration and body mass were monitored weekly and at the time of sacrifice (after 2, 3, 6 and 12 weeks of disease).

The animals were anaesthetized with pentobarbital sodium (60 mg/kg), the heart of each animal was removed, washed with ice-cold physiological saline solution (Nacl 9%), dried and processed for biochemical measurements. Homogenates were prepared on ice in the ratio 500 mg tissue for 4 ml of phosphate buffer, pH 7.5, containing 1 mmol/l Na2EDTA. For each sample 10 µl of 500 mmol/l butylated hydroxytoluene in acetonitrile was added to prevent formation of new peroxides during the assay. The homogenates were centrifuged at 20 000 x g for 15 min at 4°C and frozen at -70°C until analysis. Proteins were determined in diluted aliquots of the homogenates by the method of Lowry [15].

Chemical substances

All chemical substances were obtained from Sigma Chemical, St. Louis, USA and Sigma-Aldrich Germany. Citrate buffer 0.05mol /l pH 4,5 : 26,7 ml of A: 21,01g citric acid dissolved in 1000 ml of distilled water and 23,2 ml of solution B: 29,41g sodium citrate dissolved in 1000 ml of distilled water.

Phosphate buffer pH 7.5: NaH2PO4 10mmol/l, EDTA 1mmol/l et saccharose 250 mmol/l.

Samples analysis

The activities of of CuZn superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) were evaluated in homogenates using the spectrophotometric method (Bioxtyech kit, SOD- 525, GPx- 340 and GR-340, Oxis Rechaerch, USA). Catalase (EC 1.11.1.9) activity was assayed by the decrease in absorbance of hydrogen peroxide at 240 nm as described by Aebi [1].The G6PD activity was measured according to the procedure of Löhr and Waller [16], NADP was reduced by...
G6PD in presence of a glucose-6-phosphate, the the production of NADPH was proportionally as a formation of phosphor-glucanate acid, which measured at 340 nm. Ascorbic acid concentration was assayed according to Kyaw [14], using a simple acid phosphoglucose procedure. LPO products were determined using the method based on the reaction with N-methyl-2-phenyldimino to yield a stable chromophore with maximal absorption at 586 nm. The method was used to assay malondialdehyde together with 4-hydroxyalkenals (MDA + 4-HNE) Esterbaur et al 1991.

**Statistical analysis**

The reported data are the means of measurements and their S.E.M. values. For statistical evaluation the ANOVA two ways test followed by the Student’s t-test were applied using the Minitab 1.4 (Minitab Inc) software.

**RESULTS**

The initial mean value for serum glucose concentration was 6.39mmol/l. This level remained unchanged in the control groups but increased progressively in the diabetic animals. At the 2nd week the mean glucose concentration was 22.0 mmol/l (P< 0.01), at the 3rd and 6th weeks 32.3 and 30.1 mmol/l (P< 0.001) respectively. At the 12th week of the experiment the mean value for glucose concentration was 23.3 mmol/l (P< 0.01). There was a progressive and significant increase in body mass of control rats. The diabetic animals showed a significantly reduction in body mass, which attained 171.80 compared to control 283.48 at the 12th week (table 1). All other results are summarized in table 2 and 3.

**Table 1: Body weight and blood glucose in control and diabetic rats**

<table>
<thead>
<tr>
<th>Duration of glucose DM (weeks)</th>
<th>Body weight g</th>
<th>Blood glucose mol/l</th>
<th>C</th>
<th>D</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>246.64 ± 20.16</td>
<td>197.52 ± 24.12b 6.29.8 ± 1.2</td>
<td>22.0 ± 0.8b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>275.51 ± 34.40</td>
<td>179.78 ± 30.42a 6.91 ± 0.9</td>
<td>32.3 ± 1.5a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>253.14 ± 20.41</td>
<td>175.74 ± 32.20a 5.96 ± 0.7</td>
<td>30.1 ± 0.4a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>283.48 ± 30.35</td>
<td>171.80 ± 36.50a 6.32 ± 1.4</td>
<td>23.3 ± 0.9b</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean ± S.E.M.; n = 6 in all groups. *Significantly different from control P < 0.001; b Significantly different from control P< 0.01.

At the same time, GSH-Px activity was significantly increased. GSSG-R activity in the diabetic heart increased at the 2nd week, decreased at the 3rd and 6th weeks, then again increased above the level of the respective control group.

At the 2nd and 3rd weeks of experiment, we observed a significant decrease in the level of ascorbic acid. At the 6th and 12th weeks the concentrations of ascorbic acid were significantly higher than in the respective control groups. We found a decrease in the level of LPO products (estimated as MDA + 4-HNE) at the 2nd, 3rd and 6th weeks of diabetes. After 12 weeks of the disease the level of LPO products was significantly higher in diabetic animals than in the control group.

**DISCUSSION**

In our investigations, Cu,Zn-SOD and G6PD activity increased in the heart of diabetic animals probably to increase dismutation of superoxide anions as a compensatory activation mechanism. Similar results were obtained in other studies [11, 27, 18, 23]. G6PD is the predominant player in NADH regulation and can play an important role in an organism's overall redox status and subsequent ability to handle oxidative stress [32]. The augmentations of G6PD activity in heart of diabetic animals contribute in formation of NADH which is required in order to convert oxidized glutathione (GSSG) into GSH [33]. In addition kirkman et al [33], shown a protective effect of NADH on catalase activity.

The increase of CAT activity at the 2nd, 3rd and 12th weeks of our experiment and in other tests [11, 26, 18, 23] suggests a compensatory response to oxidative stress due to an increase in endogenous H$_2$O$_2$ production.

A significant increase in the level GSH-Px activity and a less significant one in the GSSG-R activity during the experiment confirm an efficacious defense of the diabetic heart against oxidative stress. The data obtained were similar to those presented by other authors [11, 17, 21].

Ascorbic acid is a major antioxidant that is essential for the scavenging of toxic free radicals in both blood and tissues. The disturbances in ascorbic acid metabolism in diabetes are therefore of great interest and might be important in the pathogenesis of some diabetic complications [12, 19]. The active transport of ascorbic acid appears to be decreased by hyperglycaemia and insulin deficiency. Hyperglycaemia has also been shown to inhibit the uptake of dehydroascorbic acid, the oxidized species of vitamin C [2]. In the diabetic heart we observed a decrease in the level of ascorbic acid after 3 weeks and then an increase up to the 12th week of the experiment. In a previous paper, the decreased level of ascorbic acid was found in the heart after 8 weeks of the experiment [24]. Because of an impaired balance between the generation of free radicals and antioxidant defense systems some lipid peroxidation products or degradation products may be formed [8].

Since polyunsaturated fatty acid peroxides generate malondialdehyde and 4-hydroxyalkenals, measurement of MDA and 4-HNE may be used as an indicator of lipid peroxidation. In the present work we used a method based....
on the reaction of MDA and 4-HNE with a chromogenic reagent to form a stable product with maximal absorption at 586 nm. The applied method is more specific than the most frequently used thiobarbituric acid (TBA) test because several other compounds give color products having absorption at the maximum of the TBA–MDA complex [7].

In our experiment, the heart lipid peroxidation did not increase during 6th weeks of the disease, probably due to the elevated activities of SOD and GSH-Px and to the decrease level of GSH. Similar results were reported by Parinandi et al. [21] and Tatsuki et al. [26]. Opposite results were obtained by other authors [11, 24, 13, 23].

In summary, we report significant differences between the diabetic and control groups. However, mechanisms of the defense in the heart were fairly efficacious against oxidative stress under diabetic conditions. This was demonstrated by the high levels of antioxidant compounds and the relatively low concentration of LPO products, especially during the first 12 weeks of diabetes. The tissue levels of the mentioned compounds seemed to depend on the stage of diabetes.

### Table 2: Antioxidant: parameters in the heart tissue of the control (C) and diabetic (D) rats.

<table>
<thead>
<tr>
<th>Duration of DM (Weeks)</th>
<th>CAT (µmol H₂O₂/min mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>GSH-Px (mU/mg protein)</th>
<th>GSSG-R (mU/mg protein)</th>
<th>G6PD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>D</td>
<td>C</td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>84.8±</td>
<td>103.7±</td>
<td>1.8±</td>
<td>5.2±</td>
<td>30.7±</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.9a</td>
<td>0.2</td>
<td>0.2a</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>86.7±</td>
<td>108.9±</td>
<td>1.9±</td>
<td>6.5±</td>
<td>30.6±</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>1.6a</td>
<td>0.2</td>
<td>0.4a</td>
<td>0.4</td>
</tr>
<tr>
<td>6</td>
<td>85.5±</td>
<td>63.5±</td>
<td>1.8±</td>
<td>5.1±</td>
<td>30.4±</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>1.2a</td>
<td>0.2</td>
<td>0.3a</td>
<td>0.3</td>
</tr>
<tr>
<td>12</td>
<td>89.6±</td>
<td>105.4±</td>
<td>1.6±</td>
<td>3.9±</td>
<td>30.3±</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>1.8a</td>
<td>0.1</td>
<td>0.2b</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Values are the mean ± S.E.M.; n = 6 in all groups. *Significantly different from control at P < 0.001; **significantly different from control at P < 0.005; †Significantly different from control at P< 0.01; n not significantly different.

### Table 3: Ascorbic acid and LPO products concentration in the heart tissue of the control (C) and diabetic (D) rats.

<table>
<thead>
<tr>
<th>Duration of DM (weeks)</th>
<th>ascorbic acid nmol/g tissue</th>
<th>LPO products (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>2</td>
<td>240 ± 7</td>
<td>221 ± 3</td>
</tr>
<tr>
<td></td>
<td>36.8 ± 0.3</td>
<td>24.3 ± 0.8</td>
</tr>
<tr>
<td>3</td>
<td>239 ± 6</td>
<td>207 ± 2</td>
</tr>
<tr>
<td></td>
<td>36.9 ± 0.3</td>
<td>16.2 ± 1.5</td>
</tr>
<tr>
<td>6</td>
<td>246 ± 5</td>
<td>298 ± 4</td>
</tr>
<tr>
<td></td>
<td>36.8 ± 0.3</td>
<td>17.4 ± 0.4</td>
</tr>
<tr>
<td>12</td>
<td>242 ± 6</td>
<td>267 ± 5</td>
</tr>
<tr>
<td></td>
<td>37.1 ± 0.5</td>
<td>61.4 ± 0.9</td>
</tr>
</tbody>
</table>

Values are the mean ± S.E.M.; n = 6 in all groups. *Significantly different from control at P < 0.001; **significantly different from control at P < 0.005; †Significantly different from control at P< 0.01.
REFERENCES

