Hyperglycaemia, stress oxidant, liver dysfunction and histological changes in diabetic male rat pancreas and liver: Protective effect of 17β-estradiol

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ABSTRACT

Oxidative stress is thought to play a crucial role in the pathogenesis of chronic diabetic complications. We investigated the protective effects of 17β-estradiol (E2) on alloxan-induced stress oxidant, hepatic dysfunction and histological changes in male rats liver and pancreas. Our results showed that 17β-estradiol could attenuate the increase of blood glucose in plasma and normalise the hepatic glycogen level. In addition, E2 enhanced superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (by 207, 52 and 72%, respectively, as compared to diabetic rats), reduced lipid peroxidation in the hepatic tissue (by 54%) and improved the liver dysfunction parameters by the significant decrease of gamma-glutamyl transferase (GGT), phosphatases alkalines (PAL), lactate deshydrogenase (LDH) and aspartate and lactate transaminases (AST and ALT) activities which increased in diabetic rats. Moreover, 17β-estradiol treatment in diabetic rats protects against alloxan-induced pancreatic β-cells and hepatic cells damages.

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1. Introduction

Diabetes mellitus is a chronic metabolic disorder that continues to present a major worldwide health problem [1]. It is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action associated with chronic hyperglycemia and disturbances of carbohydrate, lipid, and protein metabolism. As a consequence of the metabolic derangements in diabetes, various complications develop including both macro- and micro-antioxidant dysfunctions. In diabetes, several features appear including an increase in lipid peroxidation, alteration of the glutathione redox state and a decrease in the content of antioxidant enzymes [2].

The antioxidant properties of steroid hormones have been shown in different cells and tissues [3]. For example, 17β-estradiol protects cells from oxidative stress-induced cell aging [3–5]. Additionally, 17β-estradiol regulates several mechanisms that protect the mouse against oxidative stress [3]. Several studies have shown that treatment with estrogens reduces diabetic complications [6], protects from diabetes-dependent brain damage [6], and normalizes the endothelial function in diabetes [9]. In spite of all the evidence about its
beneficial effects, little is known about the role of 17β-estradiol in the diabetic rats and its antioxidant capacities. The present paper was carried out to study the effect of 17β-estradiol on hepatic lipid peroxidation, enzymatic antioxidants, cells damage biomarkers and histological changes in rats liver and pancreas with alloxan-induced diabetes compared to insulin.

2. Materials and methods

2.1. Animal

Adult male Wistar rats, weighing 180 ± 20 g, and obtained from the Central Pharmacy, Tunisia, were employed in the study. The animals were kept in an environmentally controlled breeding room (temperature: 20 ± 2 °C, humidity: 60 ± 5%, 12-h dark/12-h light cycle). All rats had free access to tap water and fasted overnight before blood and tissue collection. The handling of the animals was approved by the local Ethical Committee for the correct Care and Use of Laboratory Animals. Diabetes was induced in rats by a single intraperitoneal injection of freshly prepared alloxan solution in normal saline at a dose of 180 mg/kg body weight [10]. The feeding experiment was carried out for a period of 4 weeks after the induction of diabetes in 5 days (characterized by the presence of glucosuria). The rats were divided into four groups consisting of 10 rats each. Group 1 (normal control) consisted of normal rats. Group 2 served as positive control (diabetic control). Groups 3 and 4 rats were given insulin or 17β-estradiol daily: insulin; 0.5 IU/rat by intraperitoneal injection [11] and 17β-estradiol (1 μg/kgbw daily) by oral gavage [12] respectively. After 4 weeks, the animals were sacrificed by decapitation, and the trunk blood collected. The serum was prepared by centrifugation (1500 g, 15 min, 4 °C) and the pancreas and liver were removed, cleaned of fat and weighed; all these samples were stored at −80 °C until used. Pieces of pancreas were fixed in a Bouin solution for histological study.

2.2. Biochemical measurements

After the homogenisation of liver in a phosphate buffer (1 g/2 ml), the lipid peroxidation in the liver of control and E2-treated rats was measured by the quantification of thiobarbituric acid reactive substances (TBARS) determined by the method of Buege and Aust [13]. The activity of superoxide dismutase in the liver of control and treated rats was assayed by the spectrophotometric method of Marklund and Marklund [14]. The activities of glutathione peroxidase and catalase were measured by the modified method of Pagila and Valentine, and Aebi [15,16], respectively. The level of total protein was determined by the method of Lowry et al. [17]. The activity of gamma-glytamyl transferase (GGT), phosphatases alkalines (PAL), lactate dehydrogenase (LDH), aspartate and lactate transaminases (AST and ALT) in serum were measured using commercial kits from Sigma Munich (Munich, Germany) and Boehringer-Mannheim (Mannheim, Germany).

For histological studies, pieces of pancreas and liver were fixed in a Bouin’s solution for 24 h, and then embedded in paraffin. Sections of 5 μm thickness were stained with hematoxylin–eosin and examined under Olympus CX41 light microscope.

2.3. Statistical analysis

Data are presented as means ± S.D. The determinations were performed from ten animals per group and the differences were examined by the one-way analysis of variance (ANOVA) followed by the Fisher test.

3. Results

3.1. Blood glucose and hepatic glycogen

The blood glucose and hepatic glycogen levels of rats are presented in Table 1. The blood glucose level of diabetic rats increased by 167% (p < 0.001) compared to the control animals. In insulin or in estradiol (E2) treated rats, a decrease in blood glucose by 48 and 34% was observed respectively (p < 0.01; p < 0.05). The hepatic glycogen level increased significantly only in insulin-treated rats (p < 0.001).

3.2. Hepatic TBARS levels

Fig. 1 Hepatic TBARS levels in diabetic 17β-estradiol and insulin-treated rats (1.003 ± 0.11 and 1.12 ± 0.04 nmol/mg proteins, respectively) significantly decreased by 54 and 49% compared to the diabetic animals (2.2 ± 0.22 nmol/mg proteins).

3.3. Hepatic SOD, CAT and GPX activities

The activities of enzymatic antioxidants of all animal groups are presented in Fig. 2: in diabetic rats liver, a significant decrease in the activities of SOD (10.45 ± 1.25 U/mg pro-

<table>
<thead>
<tr>
<th>Table 1 – Effects of 17β-estradiol and insulin treatments on plasma glucose (mg/dl) and hepatic glycogen (mg/g) in diabetic rats</th>
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<tr>
<td>Groups</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Diabetic</td>
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<tr>
<td>Diabetic + Ins</td>
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<td>Diabetic + E2</td>
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The blood and hepatic glycogen levels of diabetic rats were significantly different from the non-diabetic rats (p < 0.001, p < 0.01). After insulin or estradiol treatment, a significant decrease in blood glucose was observed (p < 0.05; p < 0.01). The hepatic glycogen level increased only in insulin-treated rats. Data represent mean ± S.D. (n = 10). Statistical analyses as in the legend of Fig. 1.

* p < 0.05 as control rats.
** p < 0.01 as control rats.
***p < 0.001 as control rats.
† p < 0.05 as diabetic rats.
‡ p < 0.01 as diabetic rats.
§ p < 0.001 as diabetic rats.
¶ p < 0.05 as diabetic rats treated with insulin.
## p < 0.001 as diabetic rats treated with insulin.
Fig. 1 – Effects of 17β-estradiol and insulin treatments on the hepatic TBARS levels in diabetic rats. In diabetic rats, a significant \( (p < 0.001) \) increase in hepatic TBARS levels was observed. In diabetic rats treated with 17β-estradiol or insulin, an ameliorative effect was noted. Data represent mean ± S.D. \((n = 10)\); * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) as control rats; + \( p < 0.05 \), ++ \( p < 0.01 \), +++ \( p < 0.001 \) as diabetic rats; # \( p < 0.05 \), ## \( p < 0.01 \), ### \( p < 0.001 \) as diabetic rats treated with insulin.

proteins), CAT \((22.16 ± 0.83 \mu\text{mol H}_2\text{O}_2/(\text{min mg proteins}))\) and GPX \((0.47 ± 0.04 \mu\text{mol GSS/(min mg proteins)})\) by 53, 20 and 40% was observed, respectively, compared to normal rats \((22.29 ± 1.17 \text{U/mg proteins}; 27.99 ± 0.54 \mu\text{mol H}_2\text{O}_2/(\text{min mg proteins})\) and \(0.78 ± 0.05 \mu\text{mol GSS/(min mg proteins)}, \) respectively). Treatment with 17β-estradiol or insulin resulted in a significant restoration of all these enzymes and the effect was more pronounced in the group of rats treated with estradiol.

3.4. Plasmatic LDH, GGT, PAL, AST and ALT activities

The LDH, PAL, AST, ALT and GGT activities in diabetic rats were significantly higher than normal control rats by 42, 41, 49, 49 and 90% respectively. When diabetic rats were treated with 17β-estradiol \((1 \mu\text{g/kg bw daily})\) or insulin \(0.5 \text{IU/rat})\), a significant decrease of these activities was observed, in comparison to untreated diabetic rats (Table 2).

3.5. Histological results

Based on HE stained tissue sections, alloxan administration elicited severe injury of pancreatic \(\beta\) cells, such as the decrease of the islets cells’ numbers, damage and cells death (Fig. 3B) compared with normal rats (Fig. 3A). Administration with insulin \(0.5 \text{IU/rat})\) showed no significant effect on reducing the injuries of rat pancreas (Fig. 3C). The damage in \(\beta\) cells was partially reversed in 17β-estradiol-treated diabetic group (Fig. 3D).

In liver tissues, lipid accumulation in liver cells was clearly observed in diabetic rats and diabetic rats treated with insulin (black stained in the figure) (Fig. 4B and C) as compared to nor-

Fig. 2 – Effects of 17β-estradiol and insulin treatments on hepatic SOD, CAT and GPX activities in diabetic rats. In diabetic rats, after 17β-estradiol or insulin treatment, an increase in SOD, CAT and GPX occurred and the effect was more pronounced in the group of rats treated with 17β-estradiol. Data represent mean ± S.D. \((n = 10)\). Statistical analyses as in the legend of Fig. 1.
Table 2 – Effects of 17β-estradiol treatments on plasmatic AST, ALT, LDH, PAL and GGT activities in normal control, diabetic control and 17β-estradiol-treated rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
<th>LDH (U/l)</th>
<th>GGT (U/l)</th>
<th>PAL (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>135.4 ± 9</td>
<td>62 ± 6.7</td>
<td>1242 ± 61</td>
<td>2.61 ± 0.4</td>
<td>343 ± 51</td>
</tr>
<tr>
<td>Diabetic</td>
<td>193.5 ± 6***</td>
<td>88 ± 5.9***</td>
<td>1874 ± 87***</td>
<td>5.6 ± 0.5***</td>
<td>649 ± 67****</td>
</tr>
<tr>
<td>Diabetic + Ins</td>
<td>154.8 ± 18***</td>
<td>67 ± 3*</td>
<td>1602 ± 107***</td>
<td>4.2 ± 0.58*</td>
<td>486 ± 44***</td>
</tr>
<tr>
<td>Diabetic + E2</td>
<td>166.8 ± 5***</td>
<td>69 ± 6.4*</td>
<td>1195 ± 71***</td>
<td>4 ± 0.51*</td>
<td>478 ± 61****</td>
</tr>
</tbody>
</table>

The activities of AST, ALT, LDH, GGT and ALP significantly increased in diabetic rats compared to controls (p < 0.001; p < 0.001; p < 0.01; p < 0.05; p < 0.001 respectively). These activities decreased significantly after insulin or E2-treatment. Data represent mean ± S.D. (n = 10). Statistical analyses as in the legend of Fig. 1.

* p < 0.05 as control rats.
** p < 0.01 as control rats.
*** p < 0.001 as control rats.
+ p < 0.05 as diabetic rats.
++ p < 0.01 as diabetic rats.
+++ p < 0.001 as diabetic rats.
+ p < 0.05 as diabetic rats treated with insulin.
++ p < 0.01 as diabetic rats treated with insulin.

Fig. 3 – Histological comparison of the pancreas from normal controls (A), diabetic (B), 4-week insulin-treated (C) and 4-week 17β-estradiol-treated (D) rats. In diabetic rats, a decrease in pancreatic β-cells was clearly observed. A protective effect was observed only after E2-treatment. Examinations were carried out at ×400.
Fig. 4 – Histological comparison of the liver from normal controls (A), diabetic (B), 4-week insulin-treated (C) and 4-week 17β-estradiol-treated (D) rats. After E2-treatment, an ameliorative effect was observed. Examinations were carried out at the ×100.

Fig. 4A). In diabetic rats treated with 17β-estradiol, rare fat was observed in liver (black stained) (Fig. 4D).

4. Discussion

Our results showed that alloxan administration produced a rise in blood sugar. The disorder of glucose metabolism in diabetes is mainly attributed to diabetic oxidative stress due to several factors. The hyperglycemia produced by alloxan leads to the over-production of free radicals, the inactivation of the antioxidant enzymes by the non-enzymatic glycation of proteins and exerts deleterious effects on the function of pancreatic β-cells [18–20]. Hyperglycaemia also degrades antioxidant enzyme defenses by allowing reactive oxygen species to damage cells and tissues. Our study showed that hyperglycaemia is accompanied with the increase of lipid peroxidation as evidenced by the significant increase in hepatic lipid peroxidation (TBARs), the decrease of hepatic antioxidant enzymes activities and the increase in the plasmatic AST, ALT, LDH and PAL activities. These findings are in agreement with several reports. For instance, Jyoti et al. [21] reported that alloxan induced the inhibition of SOD and CAT activities and increased LPO in rat erythrocytes.

As a strategy to counteract the negative effect of oxidative stress, antioxidant-based therapy is promising to minimize the complications associated with oxidative stress in diabetes mellitus [22,23]. Recent observations have shown that many of these complications are diminished upon supplementation with certain dietary antioxidants and bio-molecules such as testosterone [24]. The use of other bio-molecule non-nutrient antioxidants such as estrogens has been reported with the same advantage [25–27].

The non-genomic antioxidant property of 17β-estradiol is due to the free phenolic hydroxyl group on the A-ring of the steroid. The direct free radical-scavenging by phenolic antioxidants has been thought to cause the interruption of free-radical chain reactions by rapidly donating the hydrogen atom of the phenolic OH to radicals [28], thus protecting pancreatic and hepatic cells from lipid perox-
idation and various other damages in cells proteins and DNA.

In addition, estrogens have been implicated in antioxidant response element (ARE)-mediated gene transcription that is the upregulation of SOD and GPX in hepatic cells [5] and other antioxidant genes: phase II detoxification enzymes, glutathione S-transferase and quinone reductase gene [29–31]. Estrogens induce antioxidant genes through the ERα or ERβ-mediated transactivation of ARE. Other works seem to indicate that 17β-estradiol is responsible for the increase in insulin secretion and the modulation of the insulin receptors [32,33], thus normalising glycaemia and defending glucose-induced liver toxicity. Estrogens also exert anti-apoptotic effects on pancreas and liver cells by the increase of the expression of the anti-apoptotic Bcl-2 and Bcl-xL, thus protecting pancreatic β cells and hepatic cells from apoptosis and therefore death [25,34,35]. All these beneficial effects of 17β-estradiol could normalise glycaemia and restore the liver function as observed in this study. Many of these pharmacologic features of 17β-estradiol are due to its potent antioxidant action. 17β-estradiol acts as an antioxidant at both prevention and intervention levels. The prevention of free radicals formation by 17β-estradiol may occur through its ability to scavenging free radical generation reactions [36], and through its activation effect on several protein-kinases like ERK, p38, and JNK [37]. In diabetes mellitus, various agents reduce oxidative stress indirectly by lowering blood glucose level and preventing hypoinsulinemia directly by acting as free radical scavengers. For example, testosterone protects from apoptotic damage induced by streptocyn in rat pancreas [24]. There have been four suggested mechanisms explaining the hypoglycaemic effect of 17β-estradiol on diabetes: (i) anti-apoptotic damage induced by alloxan in rat pancreas β-cells, (ii) estrogens decrease the stress oxidant induced by alloxan in pancreas cells by the increase of the cellular antioxidant defense system [4], (iii) to scavenge free radical produced by alloxan in liver and pancreas [30], and (iv) modulate the insulin receptors sensitivity [32].

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