Introduction

Diabetes mellitus is a chronic metabolic disorder that continues to present a major worldwide health problem. 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 including macro- and micro-antioxidant dysfunctions develop [1].

In diabetes, several features including an increase in lipid peroxidation, alteration of the glutathione redox state and a decrease in the content of antioxidant enzymes appear [2]. The antioxidant properties of steroid hormones have been shown in different cells and tissues. A number of studies have suggested that estrogens have a profound modulating effect on systemic glucose homeostasis [3, 4].

Several studies have shown that treatment with estrogen reduces diabetic complications [5], and normalizes the endothelial function in diabetes [6].

Eestrogen receptors are present in islets of Langerhans [7] and the effects of 17β-estradiol in some physiological aspects of the islet of Langerhans have been known for a long time [8]. In spite of this, the mechanism of action employed by 17β-estradiol is still largely unknown [9].

Aim of the work

The first aim of the present work was to study the effect of E2 in a diabetic rat model, to gain a better understanding of the potential protective effect of exogenous estrogens on hyperglycemia.

Effects of estrogen on hyperglycemia and liver dysfunction in diabetic male rats

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Abstract: Objective: To study the possible beneficial effect of estrogen (17β-estradiol E2) on hyperglycemia, oxidative stress and liver dysfunctions in STZ-induced diabetic rats. A total of 40 albino male rats were randomly divided into four groups: a control group (I), a diabetic group (II), a group given 17β-estradiol (E2) for 15 days (III), and a diabetic group given E2 for 30 days (IV). Diabetes was induced in the rats by 65 mg/kg streptozosin (STZ) via an intraperitoneal (i.p.) injection. E2 was given in a dose of 500μg/kg/day by oral gavage. Results: E2 administration significantly lowered plasma glucose levels, increased plasma insulin levels, and improved glucose tolerance of groups III and IV. In addition, E2 enhanced glutathione peroxidase (GPX) and reduced lipid peroxidation in the hepatic tissues (as compared to diabetic rats). E2 caused significant decrease of plasmatic phosphatase alkaline (PAL), lactate dehydrogenase (LDH), aspartate and lactate transaminases (AST and ALT) activities of group III and IV compared to group II. Moreover, E2 restored the histological structure of the liver and pancreas of treated groups and increased the insulin receptors expression in the liver of groups III and IV compared to diabetic rats. Notably, these beneficial effects of E2 on diabetic rats were more prominent in group IV compared to those of group III. Conclusion: E2 has a beneficial effect on hyperglycemia, oxidative stress and ameliorates the liver dysfunction in diabetic rats and these effects may be mediated through stimulating β-cell proliferation in pancreas and increased the insulin receptor expression in the liver tissues.

Keywords: 17 β -estradiol, diabetes, insulin, glucose tolerance, liver-male rat
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mia, hepatic lipid peroxidation, enzymatic antioxidants, and histological changes of liver and pancreas in STZ-induced diabetes. And the second aim is to discover their possible mechanism(s) of actions.

Materials and methods

Forty adult male white albino rats weighting 200-220 gm provided by the Institutional Animal Care and Faculty of Medicine, University of Assiut were included in the experiment. The experimental protocol was approved the Ethical Committee of by Faculty of Medicine, Assiut University, Egypt. The experiment was performed after a stabilization period in the laboratory for several days. All rats were housed in a room with controlled temperature (22°C ± 2°C), humidity (50% ± 5%), and a 12-hour light/dark cycle and were fed on chow and water ad libitum.

Grouping and diabetes induction

The rats were divided into four groups consisting of 10 rats each. Group I: normal control rats. Group II: diabetic rats. Groups III: diabetic rats were treated daily with 17β-estradiol (Steraloids, Inc., Newport, RI) 500ug/kg/day by oral gavage dissolved in 1% methylcellulose [10] for 15 days. Group IV: diabetic rats were treated with E2 for 30 days.

Diabetes was induced in overnight fasted rats (16 h) by intraperitoneal injection (i.p.) of a single dose of STZ “Sigma Chemical Co., St. Louis, MO” 65mg/kg dissolved in 10 mM citrate buffer (pH 4.5). Then, after 4-5 days of STZ injection, rats were screened for blood glucose levels. Rats with a serum postprandial glucose level of 180-300 mg/dl were considered as mildly diabetic and were included in the experiment [11].

Plasma glucose and insulin levels

During the experiments, the body weight, the plasma glucose and insulin levels of each animal were measured everyday for all groups.

The plasma glucose levels were determined daily using the Autokit Glucose Test Kit (Wako Diagnostics, Richmond, VA). The animals were not fasted before they received blood glucose measurements. Plasma insulin level was also determined, by using the Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem, Inc., Downers Grove, IL).

Test of oral glucose tolerance

A glucose solution (2 g/kg) was orally administered to 4-hour-fasted rats, and blood samples were taken at 0 (before glucose loading), 30, 60, 90 and 120 minutes after glucose loading. The serum glucose levels were measured before loading and 30, 60, 90 and 120 minutes after loading. The serum insulin levels were also measured at 10 min after glucose loading to examine early insulin secretion.

At the end of the experiment, all rats were euthanized by decapitation. After decapitation of the rats, blood samples were collected from retro-orbital vein in the heparin-containing tubes and were immediately centrifuged. The plasma was separated and stored at -20°C until analyzed for basal glucose and insulin levels of all studied groups [12].

Biochemical measurements

In plasma samples of all studied groups, the activity of phosphatase alkaline (PAL), lactate dehydrogenase (LDH), aspartate and lactate transaminases (AST and ALT) were measured using commercial kits from Sigma Munich (Munich, Germany) and Boehringer-Mannheim (Mannheim, Germany).

Livers from all groups were homogenized in a phosphate buffer (1gm/2ml). In the liver homogenates of all studied groups, the lipid peroxidation 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 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 [15], and Aebi [16], respectively. The level of total protein was determined by the method of Lowry et al [17].

Pathological examination

Immediately after euthanasia, pancreas and liver specimens were fixed immediately in 10% buffered formalin, embedded in paraffin, prepared as 5-μm-thick sections and stained with
hematoxylin and eosin (HE). Stained sections were examined under light microscope (Olympus CX31, Japan) and photographed using digital camera (Olympus, Camedia C-5060, Japan).

Immunohistochemical analysis

The slides from spleen and liver of all experimental groups were deparaffinized with three changes of xylene and rehydrated through a graded ethanol series to distilled water. Antigen retrieval was performed by placing the slides in 10 mM citrate buffer (pH 3.0), heating them in a microwave oven for 20 min, and allowing them to cool to room temperature for 20 min. Slides were rinsed once with phosphate buffered saline (PBS), and the endogenous peroxidase activity was blocked by incubating the samples for 30 min in the blocking solution (3% H2O2 in PBS), followed by rinsing three times with PBS. Non specific binding was blocked by incubating the slides for 30 min in PBS containing 2% normal goat serum (Vector Laboratories, Burlingame, CA) and 1% Triton X-100, followed by incubation with specific antibodies for insulin receptors.

Statistical analysis

Data are expressed as mean ± SE for all parameters. The data were analyzed using Graph Pad Prism data analysis program (Graph Pad Software, Inc., San Diego, CA, USA). For comparison of statistical significance between different groups Student Newman-Keuls t-test for paired data were used. For multiple comparisons, one-way analysis of variance (ONE-WAY ANOVA) test followed by the least Significant Difference (LST). Correlations were assessed using Spearman’s non-parametric correlation coefficient δ as described by Knapp and Miller [18]. A value of P ≤ 0.05 was considered statistically significant.

Results

In this study, the effect of 17β estradiol (E2) administration on hyperglycemia and its detrimental effects on pancreas and liver in a diabetic rat model that was induced by i.p. injection of 65mg/Kg of freshly prepared STZ were evaluated.

Effect of E2 on plasma glucose levels and other diabetic manifestations in STZ treated rats

Table 1 showed that the mean plasma glucose levels of group II was significantly higher than those of three studied groups (p<0.001). When STZ-induced diabetic animals were treated with a dose of E2 for 15 days, the mean plasma glucose levels was decreased but still significantly higher than those group I and IV (p<0.01 respectively). However, the mean plasma glucose levels of group IV were decreased but still significantly higher than those of group I (p<0.05).

The insulin levels of group II were decreased significantly than those of the other studied groups. The insulin levels of group III increased but still significantly lower than those of control group. Prolongation of treatment of group IV with E2 increases the insulin levels significantly more than those of group III but still significantly lower than those of control group (Table 1).

On the other hand, the daily food intake of animals treated with STZ was markedly higher compared to the other studied groups (p<0.001).The food intake of group III was significantly higher than those of control animals and group IV (p<0.01 and p<0.05 respectively). Treatment of these diabetic animals with E2 for 30 days markedly suppressed their food intake (Table 1).

In spite of increased food intake of group II, there was a lack of weight gain in this group,
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which is a characteristic clinical feature of diabetes. Treatment of diabetic rats with E2 for 15 and 30 days resulted in significant increase in final body weights as compared to their initial values (p<0.01 and p<0.001 respectively) (Figure 1A).

Group III had a significantly reduced final body weight compared to the control animals, and IV group (p<0.01). The final body weight of group IV was significantly lowered than those of group I (p<0.05) (Figure 1B).

Effect of E2 treatment on oral glucose tolerance test

To functionally assess the degree of β-cell function in different groups, the oral glucose tolerance test was performed to further assess glucose and insulin response after glucose loading. Figure 2A shows that the plasma glucose levels in group II were dramatically increased after oral glucose loading, which were significantly higher than the levels in control rats, group III and IV (p<0.001) and they remained at above 290 mg/dl at 120 min after the glucose loading. Treatment of group III with E2 attenuated the increase in plasma glucose levels compared to the levels of group II (p<0.05) but the increase of plasma glucose of group IV was insignificantly different from the levels of group I.

In addition, analysis of the plasma insulin level during the first 60 min following oral glucose
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loading was determined. Plasma insulin levels of each group during the oral glucose tolerance test were calculated as percent increase based on the initial concentration levels of 1.95 ng/ml for the control group, 0.76 ng/ml for group II, and 1.67 ng/ml for the group III group and 1.8 ng/ml for the group IV (Figure 2B). The level of insulin was increased by 400% at 30 min in control animals, while there was no clear increase of insulin level in diabetic mice (group II). In contrast, the level of insulin in E2-treated animals (group III) was increased by 224.1% (P<0.05).

The glucose tolerance test reveals that while the STZ-induced diabetic rats almost completely lose the ability to secrete insulin in response to glucose loading, treatment with E2 for 15 and 30 days restores some of the ability to secrete insulin.

Effect on hepatic TBARS and GPx levels

Figure 3A showed the effect of E2 treatment on the hepatic glutathione peroxidase (GPx) activity. The levels of GPx activities of group II were significantly decreased compared to group I, III and IV (p<0.001, p<0.05 and p<0.001 respectively). E2 treatment for 15 days for group III significantly increases the level of mean GPx activity but it was still lower than those of group I and IV (p<0.05).

However E2 treatment for 30 days for group IV significantly increased the GPx activity levels toward the normal levels of group I, as there were no significant differences between their levels.

The mean TBARS levels of group II were significantly higher than those of group I, III, IV (p<0.001). The mean TBARS levels of group III were significantly higher than those of group I and IV (p<0.01). However these levels of group IV were not significantly different from those of group I (Figure 3B).

Effect of E2 treatment on plasmatic LDH, GGT, PAL, AST and ALT activities

In addition, the effect of E2 treatment on plasma levels of AST, ALT, LDH and ALP activities in all studied groups were studied. The activities of AST, ALT, LDH and ALP significantly increased in diabetic rats compared to controls, group III and IV (p<0.001 respectively). Although the activities of AST, ALT, LDH, of group III were significantly higher than those of group I and IV (p<0.01). They were nearly normal in group IV after E2 treatment for 30 days as there were no significant difference between the mean plasma levels of these activities of group IV and group I (Figure 4A, B and C).

As shown in (Figure 4D), the mean plasma levels of PAL activities of group II were significantly increased than those of group I, III and IV (p<0.001). Although the mean levels of PAL

Figure 3. Effect of 17β-estradiol (E2) on the hepatic GPx (A) activity and TBARS Levels (B). ***p<0.001, **p<0.01, *p<0.05, NS: non significant as compared to group I. +++p<0.001, +p<0.05 as compared to group II. ##p<0.01, #p<0.05 as compared to group IV.
activities in group III were higher than those of group I and IV (p<0.01), while the levels of group IV were not significantly different from those of group I.

**Histopathological results**

The histopathological examination of HE-stained sections of the pancreas of STZ-induced diabetic rats showed vascular and parenchymal changes. The vascular changes were in the form of congestion, edema and thrombosis (Figure 5B and C). Fibrosis in the interstitium with infiltration of mononuclear cells such as lymphocytes was seen (Figure 5D). The parenchymal changes were observed in the pancreatic islets in STZ-induced diabetic rats where cells were destroyed and underwent shrinkage (Figure 4E), when compared with islets of control animals (Figure 5A). After 15 days of treatment the islets returned to its normal appearance except some lymphocytic infiltration were seen (Figure 5F). After 30 days, the whole examined tissue in the pancreas resembles the control one. Also, proliferation of the beta cells were noticed (Figure 5G).

Examination of the liver of STZ-induced diabetic rats revealed vaculation of the hepatocytes (Figure 6B), focal and diffuse Kupffer cell proliferation (Figure 6C), coagulative necrosis of the liver cells with mononuclear infiltration (Figure 6D), when compared with control liver (Figure 6A). After 15 days, the liver showed vaculation of the hepatocytes and proliferation of the Kupffer cells (Figure 6E). After 30 days of treatment, the liver underwent regeneration and Kupffer cell proliferation were also seen (Figure 6F).

**Immunohistochemistry**

Immunohistochemical results of insulin receptor in the liver revealed positive insulin reaction in the control rats and moderate expression of insulin receptors after 15 days of treatment. After 30 days, the insulin receptor expression resembles the control pancreas. STZ-induced
diabetic rats showed negative insulin receptor (Figure 7).

Discussion

In the present study, the effect of 17 β-estradiol (E2) treatment in a diabetic rat model is studied, to gain a better understanding of the potential protective actions of an endogenous estrogen as well as the mechanism of its actions.

In this study, the blood glucose levels of animals with mild diabetes (blood glucose ranging from 180 to 300 mg/dl) were significantly reduced after estrogen treatment in group III and IV compared to untreated group, yet, their improvement was not to such an extent that their values were back to normal. The hypoglycemic effect of E2 in this study was attributable to an improved glucose tolerance of group III and IV, and this may result from an improved ability of the pancreas to release insulin following oral glucose loading as the amount of insulin of group III and IV were significantly increased than those of group II. This finding is consistent with results obtained from Yambe et al [19] who reported that treatment of diabetic rats with E for 15 days decreased the blood glucose levels but not back to normal.

Mechanistically, it was suggested in earlier studies that E2 has a direct insulinotropic effect by blocking the ATP-sensitive potassium channels present in the cytoplasmic membrane of pancreatic β-cells [20].

The mean plasma levels of insulin of diabetic rats were significantly decreased than those of groups III and IV. It is suggested that the elevated levels of sex steroid hormones (particularly estrogens) during pregnancy may stimulate the proliferation of islet β-cell growth, which then results in increased insulin synthesis and release [21].

Our results are in consistence with Liu and Mauvais-Jarvis [22] who reported that all positive changes towards better glycemic control could

be attributed to the roles played by estradiol both at β-cells of the pancreas as well as peripheral insulin-sensitive tissues.

The beneficial effects of E2 on pancreas function were supported by our histological findings which demonstrated that E2 treatment of group III was accompanied by restoring the structure of the pancreas besides some lymphocytic infiltration, but group IV had a histological structure resembles that of control group and the results showed that E2 treatment stimulates proliferation of islet β-cells in group IV. The progressive improvement in the structure of the pancreas is seen with prolongation of period of treatment of estrogen. These results were inconsistent with those of Meier et al [23, 24]. Yambe et al [19] suggested that E2 causes proliferation of the existing β-cells which played a critical role in the renewal of the β-cell pool.

In the present study, it is noted that STZ-induced diabetic rats had a reduced body weight gain as there were no significant differences between their initial and final weight, but a significantly increased food intake compared to control animals, suggesting the presence of growth retardation due to the lack of insulin and, subsequently, the reduction of nutrient uptake by various cells in the body. In comparison, the body weight gain of animals treated with STZ+E2 was significantly increased compared to diabetic animals.

E2 treatment decreased food intake of group III to a level which was still significantly higher than those of control. But when E2 treatment lasted to a longer period in group IV, the food intake decreased to a level close to that of normal animals. This observation agrees with several earlier studies in mice, rats, and other mammals showing that E2 has a suppressive effect on food intake [25]. Mechanistically, it has been demonstrated that the estrogen's effect on feeding and body weight changes is mediated via activation of the ERα signalling pathways [26, 27]. This was also supported by findings of Yamabe et al [19] who reported that the ability of E2 to decrease food intake may partially contribute to its hypoglycemic effect. However, it should also be noted that the plasma glucose levels in animals treated with STZ +E2 (group III and IV) are lower than the levels seen in animals treated with STZ alone. Taken together, it appears that the hypoglycemic effect of E2 appears to be largely independent of its suppression of food intake.

Figure 6. Representative micrograph of the liver from control, STZ-induced diabetic and E2 treated rats. A) Control liver. B) Vacuolated hepatocytes (arrows). C) Focal Kupffer cell proliferation (asterisks). D) Coagulative necrosis of the hepatocytes with mononuclear infiltration. E) After 15 days, hepatocyte vacuolation and Kupffer cells proliferation. F) 30 days after treatment, Regeneration of the hepatocytes. HE stain. Bar= 50μm.
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This study showed that hyperglycemia in group II is accompanied with the increase of lipid peroxidation as evidenced by the significant increase in hepatic lipid peroxidation (TBARs), the decrease of hepatic GPx enzyme activities and the increase in plasma AST, ALT, LDH and PAL activities. These results were supported by those of Hamdena et al [28] who reported that hyperglycemia produced by STZ 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. In this study, treatment of diabetic rats with E2 for 15 days improved the levels of hepatic GPx and plasma antioxidants and decreased the hepatic TBARs. The improvement increased in group IV as the period of treatment lasted to 30 days. These findings were supported by the histological results in this study, as E2 treatment results in regeneration and proliferation of Kupffer cells and restored the structure of the liver in group IV nearly to normal structure.

At the peripheral insulin-sensitive tissues, estradiol is known to modulate insulin sensitivity and, consequently, glucose homeostasis. This was inconsistent with our immunohistological results which showed that the group III and IV had increased expressions of insulin receptors compared to that of STZ induced diabetic rats. The insulin receptors expression in group IV was higher than those of group III. These results were supported by the previous report that the low concentrations of 17-β-estradiol (similar to early pregnancy) could be responsible for the increase in insulin sensitivity by increasing the amount of insulin receptors in peripheral tissues [31].

This was explained by the fact that estradiol can counteract the effects of hyperglycemia-induced downstream singling of the insulin receptors, as well as modulating insulin receptors tyrosine phosphorylation and that 17β-estradiol is responsible for the increase in insulin secretion and the modulation of the insulin receptors, thus normalizing glycaemia and defending glucose-induced liver toxicity [32].

Conclusions

In conclusion, this experimental study revealed that 17 β-estradiol had beneficial effects on STZ induced diabetic rats. It has hypoglycemic effect and restore the function and structure of pancreas and liver. The effect of 17 β-estradiol on diabetes may be due to its antioxidant action as estrogens decrease the oxidative stress induced by STZ in liver cells by the increase of hepatic antioxidant defense system and scavenge of plasma free radical. In addition, the mechanism of action of E2 could be through the upregula-

Figure 7. Representative micrograph of the liver from control, STZ-induced diabetic and E2 treated rats. A) Control liver showing positive insulin receptor. B) STZ induced a decrease insulin receptor. C) Moderate expression of insulin receptor after 15 days of treatment. D) After 30 days, the insulin receptors well expressed as control liver. Bar= 50µm.

damage cells and tissues [28]. The study showed that hyperglycemia in group II is accompanied with the increase of lipid peroxidation as evidenced by the significant increase in hepatic lipid peroxidation (TBARs), the decrease of hepatic GPx enzyme activities and the increase in plasma AST, ALT, LDH and PAL activities. These results were supported by those of Hamdena et al [28] who reported that hyperglycemia produced by STZ 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. In this study, treatment of diabetic rats with E2 for 15 days improved the levels of hepatic GPx and plasma antioxidants and decreased the hepatic TBARs. The improvement increased in group IV as the period of treatment lasted to 30 days. These findings were in agreement with EL naser et al [29] who reported that estradiol treatment showed more effective role in reducing oxidative stress than do insulin treatment although none of them succeeded in getting it back to normal control values. In addition, estrogens have been implicated in antioxidant response element (ARE)-mediated gene transcription and the upregulation of SOD and GPX in hepatic cells [30].
tion of insulin receptors, by increasing the insulin receptors expression in the liver tissues. These intriguing observations raise the possibility that administration of oral E2 may be beneficial in type II diabetic patients with accelerated loss of pancreatic islet β-cells.

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