The Gene Expression of Antioxidant Enzymes in Streptozotocin-Induced Experimental Diabetes in Rat Liver Tissue

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Abstract

In this study, Wistar rats were experimentally induced diabetes and the gene expression profile of CuZn-SOD and CAT enzymes were investigated in the liver tissue of these rats. The rats were divided into two groups as control and the experimental diabetes group (DM). In group DM, as a single dose of 50 mg/kg streptozotocin (STZ) dissolved in 0.01 M citrate buffer (pH: 4.5) was given intraperitoneally to the rats. 0.01 M citrate buffer which is the vehicle for STZ was applied to the rats in control group. 72 hours after STZ treatment, blood samples were collected from the tail vein, and the blood sugar levels were measured. Rats with fasting blood sugar levels above 350 mg/dl were considered to be diabetic. The rats were decapitated 21 days after STZ treatment. The liver tissues were collected, and the gene expression profile of CuZn-SOD and CAT in the liver was measured using real-time PCR technique. In conclusion, it was found that a significant decrease was observed in the expression of CuZn-SOD and CAT genes in DM group when they were compared to that of control group.

Keywords: Rat, diabetes, streptozotocin, CuZn-SOD, CAT
Introduction

Diabetes mellitus (DM) is a disease that emerges with the insufficient proliferation capacity of the pancreatic beta (β) cells due to either loss of these cells or deterioration of their function [1]. DM is defined as a metabolic disease that occurs as a result of defects in insulin secretion or defects in the effect of insulin. It is characterized with the chronic hyperglycemia and it is a metabolic disease that leads to the deteriorations in the carbohydrate, fat, protein, water and electrolyte metabolisms [2-5]. Both the genetic and environmental factors have roles in DM [2]. It has been explained that some factors (such as; pancreatic β cell destruction and the other reasons that cause the insulin resistance) lead to different pathologies which contribute to the DM development [5]. The chronic hyperglycemia leads to damage, dysfunction and failure in organs (such as eyes, kidneys, heart) and nerves as well as blood vessels in the long-term [5].

The free radicals are known as molecules that contain unpaired electrons in their atomic or molecular orbitals. Therefore, they are regarded as extremely reactive for reactions with biological molecules [6]. It has been asserted that the free radicals due to DM cause damage in the structure of lipids, proteins and nucleic acids resulting in genetic mutations, the loss of membrane integrity and structural or functional alterations in the proteins [7]. The increase of free radicals in DM casues alterations in the antioxidant defense mechanisms thus leading to lipid peroxidation, increase in insulin resistance and further DM complications after damaging the cellular organelles and enzymes [8, 9]. Different hypotheses have been tested regarding the DM and its complications. These hypotheses were; advanced glycation end product (AGE) hypothesis, aldose reductase hypothesis, oxidative stress, real hypoxia, carbonyl stress, changes in lipoprotein metabolism, increase in protein kinase C activity, changes in growth factors and changes in cytokine activity. Particularly, the impacts of free radicals have been examined among these hypotheses [10]. Reactive oxygen species (ROS) formation and the antioxidant systems protects tissues from the damage by both intracellular and intercellular defense mechanisms. The intracellular defense is carried out by albumin, bilirubin, transferrin, ceruloplasmin and uric acid whereas the intercellular defense mechanism is conducted through superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GSH-Px), glutathione reductase (GR), catalase (CAT) and cytochrome oxidase enzymes [11]. ROS alters gene expressions and the activities of the antioxidant defense
enzymes [12,13]. Some mechanisms (such as free radicals, glycation, glycosylation, autooxidative polyol path activity, various changes in antioxidant defense system, hypoxia) increase the oxidative stress. The alterations in the glucose concentration result in both intracellular and intercellular oxidative stress [14,15]. High glucose concentrations may lead to higher mRNA levels and the higher activity of antioxidant enzymes. It has been stated that the higher expression of these antioxidant enzymes may occur against glucose-induced oxidative stress [14]. Glucose is converted into enediol radical anion in the presence of transition metals and it leads to the superoxide radical formation in the presence of molecular oxygen [14]. Superoxide radical is converted into the highly effective hydroxyl radical in the presence of metal ions [14]. Another important target of oxidative stress is pancreatic β cells. The β cell dysfunctions are caused by high blood glucose levels and/or the increase in the free fatty acid amount. It has been reported that the increased lipid peroxidation may increase glucose autooxidation in the Langerhans islet [16]. Oxidative stress leads to the apoptosis in the pancreatic β cells of pancreas in type-1 DM and it also leads to insulin resistance by preventing the insulin release mechanisms in type-2 DM [14]. The increased oxidative stress in DM causes the inactivation of the antioxidant enzymes (such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px)). On the other hand, it also triggers the β cells to be more sensitive to ROS due to the failure of the antioxidants. Therefore, high blood glucose levels can damage the liver, kidney and retina cells [16-18]. It has been well known that oxidative stress have roles in the pathology of cardiovascular diseases, neurological damage, cancer, diabetes, ischemia/reperfusion and aging [6]. It has been shown that the CuZn-SOD decreases in the erythrocytes as well as kidney and liver tissues of the diabetic rats [19]. It has been indicated that the non enzymatic glycation of antioxidant enzymes increases the oxidative stress and the CuZn-SOD is inactivated by glycation [20]. In this study, we aimed to examine the gene expression levels of the antioxidant enzymes in the liver tissue of the rats with streptozotocin (STZ)-induced DM case.
Materials and Methods

Procurement and care of the rats

An ethics committee approval was obtained from İnönü University, Faculty of Medicine Experimental Animal Local Ethics Committee, dated 03/12/2010 and with protocol no 2010/A-75. 8-10 weeks old Wistar albino rats were provided from İnönü University Faculty of Medicine Experimental Animals Reproduction and Research Center. During the tests, rats were kept in a room with 21°C, 55-60% humidity with 12 hours cycles of dark and light.

Experimental Procedure

The DM model was created according to the protocol, performed by Pushparaj et al. [21]. The weights and the fasting blood glucose levels of all rats were measured before the experiments. The rats were divided into two groups; control (C) and DM. The C group rats (n:10) were injected with a single dose of STZ-vehicle (0.01 M citrate buffer (pH: 4.5) intraperitoneally (1 ml). The DM group rats (n:7) starved overnight, one night before the single dose injection of STZ (50 mg/kg) (dissolved in 0.01 M citrate buffer (pH: 4.5)). The fasting blood glucose levels of all rats were measured after 72 hour of the injection. The blood samples were obtained from the tail veins and the glucose concentrations were detected by a glucometer. The rats with the fasting blood glucose ≥350 mg/dl were classified as diabetic. The starving blood glucose concentrations and the weight of all rats were measured before the experiments, 72 hours as well as 21 days later (on the 21st day) after the injections.

Removal of Tissues and Analysis of the Samples

At the end of the experimental procedure, animals both in group DM and group C were decapitated under xylazine-ketamine induced anesthesia (ketamine hydrochloride, Parke-Davis. Eczacibaşı, Istanbul, 75 mg/kg and 5 mg/kg xylazine hydrochloride Rompun, Bayer) and liver tissues were removed. Liver CuZn-SOD and CAT mRNA expression levels were analyzed with RT-qPCR.
RT-qPCR

Total RNA isolation was carried out from liver samples using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Total extracted RNA was run on 1% agarose gel and total mRNA was visualized with Ethidium Bromide staining over a UV transilluminator. The concentration of the purified total RNA was determined by NanoDrop 2000 UV-Vis Spectrophotometer. SuperScript® III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) was used for reverse transcription (RT) reactions and the manufacturer’s suggested protocol was applied. Equal amounts of total RNA were added into each RT reaction and oligo dT-18 primer was used to extend all containing mRNAs. CuZn-SOD, CAT [22], and GAPDH housekeeping gene primer sequences [23] were ordered (Table 1). Briefly, PCR amplification mixture (20 μl) contained cDNA, 1 μl of forward primer (10 pmol/μl), 1 μl of reverse primer (10 pmol/μl) and 10 μl of 2x SYBR Green I Master Mix (LC480 SYBR Green I Master Mix, Roche 04707516001). PCR amplification was performed by 94°C for 3 min denaturation; 60 cycles of 94°C for 30 s, 58°C for 30 s, and final heating of 72°C for 40 s. All qPCR were performed in three wells. For determination the change in CuZn-SOD and CAT gene in DM group, relative mRNA expression levels were calculated according to housekeeping gene (GAPDH) using the $2^{-\Delta\Delta Ct}$ calculation method [24]. PCR products were also run in DNA agarose gels and correct sized PCR products were obtained as 346 bp, 383 and 652 bp for GAPDH, CuZn-SOD and CAT genes, respectively (Figure 1).
Table 1: Primer sequences and expected product sizes for antioxidant enzymes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>PCR product size (bp)</th>
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<tbody>
<tr>
<td>GAPDH F</td>
<td>5’-CGTGGAGTCTACTGGCGTCT-3’</td>
<td>346&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>5’-GGATGCAGGGATGATGTCTTCT-3’</td>
<td></td>
</tr>
<tr>
<td>CuZn-SOD F</td>
<td>5’-GCAGAAGGCAAGCGGTGAAC -3’</td>
<td>383&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CuZn-SOD R</td>
<td>5’-TCACACCACAAGGCAAGC-3’</td>
<td></td>
</tr>
<tr>
<td>CAT F</td>
<td>5’-GCGAATGGAGAGGCAGTGTAC-3’</td>
<td>652&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT R</td>
<td>5’-GAGTGACGTGTCTTCATTAGC-3’</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> CuZn-SOD (copper–zinc superoxide dismutase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), CAT (catalase).

<sup>b</sup> F: forward primer, R: reverse primer

<sup>c</sup> Primer sequences were taken from Wang et. al. [23]

<sup>d</sup> Primer sequences were taken from Bhor et. al. [22]

Figure 1. The agarose gel electrophoresis image of the PCR products of GAPDH, CAT and CuZn-SOD enzymes. 100 bp DNA marker was used (Fermentas). The predicted sizes were given in Table 1.
Statistical Analyses

The 9.05 statistical package of SPSS for Windows program was used in order to analyze the data. Primarily, we tested whether our findings show normal distribution or not by using the Shapiro-Wilk test. The data for all parameters were found normally distributed (p>0.05). The data were indicated as a mean± standard error of mean (SEM) for all experimental groups. The independent sampling t-test was used in order to statistically compare the gene expression and rat body weight data. When the p value was lower than 0.05 (p<0.05), it was accepted as statistically significant. The statistical analysis of fasting blood glucose concentrations of rats was performed by using one-way analysis of variance (ANOVA). The homogeneity of the variances was tested by using Levene test (p<0.05) and the homogeneity of the variances were detected (p>0.05). The multiple comparisons between the groups were performed by using a Tukey test. When the p value was lower than 0.05 (p<0.05), it was accepted as statistically significant.

Results

Fasting Blood Glucose Values of Animals

Fasting blood glucose concentrations of DM group rats increased dramatically (p<0.05) to DM levels on day 3 and on day 21 after injections, although, it did not alter (p>0.05) in control group animals during the experimental period (Figure 2).
**Figure 2.** Fasting blood glucose concentrations of groups (mg/dl). The different letters represent the significant differences among the days (p<0.05).

**The Body Weight of Animals:** Body weight of the control group rats did not change (p>0.05) during the course of the experiment. On 21 day, the body weight of rats decreased (p>0.05) in group DM animals (**Figure 3**).

**Figure 3.** The body weights of the rats in both groups (g). The different letters represent the significant differences between the days (p<0.05).
Molecular Genetic Findings:

CAT and CuZn-SOD mRNA levels were investigated by RT-qPCR method in liver of DM model of rats. It was fond that both CAT and CuZn-SOD mRNA levels were decreased (p<0.05) in DM group rats (Figure 4).

![Figure 4](image-url)  
*Figure 4.* The levels of liver CAT, CuZn-SOD/GAPDH mRNAs of groups. The different letters represent the significant differences between the groups (p<0.05).

Discussion

In this study, we examined the gene expression levels of the CuZn-SOD and CAT antioxidant enzymes in the liver tissues of the STZ-induced experimental diabetic rats. At the end of the experimental period, DM case was successfully accomplished in DM rats. The CAT and CuZn-SOD mRNA levels of control and DM group rats were compared and a statistically significant decrease was observed in the gene expression levels of DM group rats (p<0.05).
ROS, which are produced in DM, can lead to many alterations in the antioxidant defense system. It has been specified that the production of ROS in DM causes the histological changes in liver tissue, such as nucleus degeneration, perivascular infiltration, central venous blood collection, cellular swelling [25]. ROS are produced continuously during aerobic metabolism and they are destroyed by various antioxidants. The increase in the prooxidants or the insufficiency of the antioxidants in the oxidative stress have a key role in the repair mechanism due to the excess amount of molecular damage [26]. According to several studies, the oxidative stress in DM has important impacts on the development of diabetic complications [10,27-30]. In the diabetic hyperglycemia, the radical formation increases and thus the antioxidant defense system may not function as desired, therefore, antioxidants are required for repair mechanisms [14,31,32]. The deterioration in the level of micronutrients in DM affects the enzyme activity [33]. Additionally, oxidative stress, glycation as well as the changes in the concentrations of cofactors can also lead to the alterations in the antioxidant activity [34]. Kakar et al. [35] examined the antioxidant enzyme changes in the kidney tissues of the male Sprague Dawley diabetic rats for 6 weeks. The authors showed that the total SOD and CuZn-SOD activity increased in the first week but, in the sixth week, total SOD activity decreased. The researchers have also stated that the decline in the SOD activity in the 6th week could be directly due to the H₂O₂ inhibitory effect or enzyme glycation. In the same study, the CAT activity was also shown to be increased in the 1st week and it started to decrease from the 2nd week. It has been specified that this decline could be due to the increase in amount of the superoxide radicals. In the female STZ-induced experimental diabetic Wistar rats, it has been observed that there was an increment in the lipid peroxidation due to the high degree of hyperglycemia and a decrease of glutathione levels in erythrocytes [36]. In the testes of the male STZ-induced experimental diabetic Spraque–Dawley rats, increased oxidative stress led to an increase in SOD activity but a decrease in the CAT activity [37]. It has been specified that the decrease in the SOD and CAT enzyme levels were due to the ROS radicals and possible enzyme glycation in the liver and kidney tissues of these diabetic rats [38]. Meanwhile, other studies have shown that the antioxidant enzyme gene expressions can also be affected in DM case. There was an increment in the gene expression and the activity of the SOD enzyme in the brain tissue of the STZ-induced diabetic rats [39]. Cho et al. [40] have found that the SOD and CAT enzyme activities increased in the liver tissue of the diabetic rats, however, there was no significant difference in the mRNA levels of
these these genes between the control and diabetic group rats. In another study, it was reported that the CAT enzyme activity decreased, whereas the mRNA levels of the CAT gene increased in the STZ-induced diabetic rats after 6 weeks in the kidney cortex tissue. In the same study, the mRNA levels and activity of the CuZn-SOD enzyme was shown to increase [41]. Another group of researchers also examined the mRNA expression levels of antioxidant enzymes and they stated that there was no significant change in the mRNA expressions of the CAT and GPx genes in the glomerular tissue of diabetic rats [42]. Similarly, it has been found that there was no alteration in the SOD and CAT mRNA levels in the small intestine tissue of the diabetic rats [22]. Sadi et al. [43] performed a study in which their results are similar to ours. According to their study, they have also detected that the gene expressions and the activity of CuZn-SOD and CAT antioxidant enzymes decreased and oxidative stress increased excessively in the liver tissue of the diabetic rats.

In the literature, it has been specified that the gene expression of the SOD and CAT antioxidant enzymes decreased [43-45], increased [39-41] or even it did not change [22, 40, 42]. In this study, the decrease of CAT and CuZn-SOD mRNA levels in rat liver tissue could be due to free radical formation, glycation, changes of cofactors and degradation of micro nutrient levels [34]. Liver is an organ with a central metabolic importance and it is damaged in DM via free radicals [46]. The excessive accumulation of H₂O₂ can be converted to hazardous OH⁻ radicals by Fenton/Haber–Weiss reaction [34,47,48]. These OH⁻ radicals have been shown to damage the DNA molecule [49]. The DNA damage in the liver tissue due to DM [50] may prevent the gene expression. Nuclear factor κ-B (NF-κB) is sensitive to oxidative stress and it regulates many gene transcriptions related to immunity and inflammation [51,52]. NF-κB can be activated by various inducers (such as bacterial endotoxin, TNF-α, IL-1β, mitogens, viral proteins, ionized radiation, UV light and some chemicals) in cells. It has been widely accepted that the ROS production is related to NF-κB activation. The agents (including endotoxins, TNF-α, IL-1 and ionized radiation) that activate the NF-κB in cells lead to oxidative stress [51]. It has been known that the Advanced Glycation End-products (AGE) formation and its accumulation increase with an accelerated rate in the diabetic status. It has been proven that AGE induces the inflammatory responses that cause NF-κB production and it leads to oxidative stress [53]. Oxidative stress can act as signal transducers and thus decrease the gene expressions of the enzymes via a NF-κB pathway [54]. The excess amount of oxidative stress can decrease the enzyme activity since
they affect both the transcription and the translation of these enzymes [43]. In conclusion, we believe that the decreased antioxidant enzyme gene expressions can be reversed by increasing the levels of antioxidants. Therefore, it is possible to prevent the DM induced oxidative stress by the elevated levels of these enzymes.

References


The Gene Expression in Experimental Diabetes


