Biochemistry
Research Article – 57060

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The effects of crocin (active contstituent of saffron) treatment on brain antioxidant enzyme mRNA levels in diabetic rats

Diyabetik ratlarda safranın aktif içeriği olan krosin tedavisinin beyindeki antioksidan enzimlerin mRNA seviyeleri üzerine etkisi

DOI 10.1515/tjb-2016-0018
Received September 11, 2015; accepted December 30, 2015

Abstract: Objective: The aim of the present study is to evaluate the effect of crocin on mRNA expression of antioxidant enzymes, SOD, CAT and GPX in the brain of the STZ induced diabetic rats.

Methods: Thirty animals randomized in three groups containing ten animals in each group as follows; control (non-diabetic rats), DM (STZ-induced untreated diabetic rats), DM+crocin (STZ-induced diabetic rats treated with crocin,). Crocin was given at a dose of 20 mg/kg bw/day by gavage for 21 days.

Results: STZ injection caused a significant increase in mRNA expression of antioxidant enzymes, SOD, CAT and GPX when compared to control group. Crocin given to diabetic rats significantly decreased mRNA expression of antioxidant enzymes, SOD, CAT and GPX when compared to DM group.

Conclusion: The present study demonstrates that crocin can modulate mRNA expression of antioxidant enzymes, SOD, CAT and GPX and oxidative stress in the brain of the STZ induced diabetic rats.

Keywords: Crocin, antioxidant enzyme genes, brain, diabetes mellitus, mRNA

Özet: Amaç: Bu çalışmanın amacı, STZ ile oluşturulan diabetik ratların beyin dokularındaki SOD, CAT ve GPX antioksidant enzimlerin mRNA gen ekspresyonları üzerine krosinin etkilerini ortaya koymaktır.

Metod: Random olarak belirlenen 30 rat her grupta 10 adet olacak şekilde şu şekilde belirlenmiştir; Kontrol (Diyabet olmayan grup), DM (STZ ile diyabet yapılan grup), DM+Krosin (STZ ile diyabet yapılan ve krosin ile tedavi edilen grup). Krosin 20 mg/kg/gün dozunda 21 gün süreyle gavajla uygulanmıştır.

Bulgular: STZ enjeksiyonu kontrol grubuyla karşılaştırıldığında antioksidant enzimler olan SOD, CAT ve GPX'in mRNA ekpresyonlarında dikkat çekici bir artışa sebep olmuştur. Diyabetik ratlara krosin verildiğinde SOD, CAT ve GPX'in mRNA ekpresyonlarında dikkat çekici bir düşüşe sebep olmuştur.

Sonuç: Çalışmamız STZ ile oluşturulan diyabetik ratların beyin dokularında krosin'in SOD, CAT ve GPX enzimlerinin mRNA ekpresyonlarını ve oksidatif stresi düzenlediğini ortaya koymuştur.

Anahtar Kelimeler: Krosin, antioksidant enzim genleri, beyin, diabetes mellitus, mRNA

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Introduction

Diabetes mellitus (DM) is a metabolic disease, which is defined as a group of disorders characterized by hyperglycemia and deterioration of carbohydrate, lipid and protein metabolisms. DM leads to increased oxidative stress and impaired antioxidant defense systems, which result in the onset and progression of diabetes-related complications [1]. DM could initiate degenerative processes in brain and several peripheral organs following hypoxic/ischemic events [2].

Oxidative stress occurs as a result of an imbalance between oxidants and antioxidants in favor of oxidants. Chronic exposure to hyperglycemia is one of the several reasons for the increased oxidative stress levels in diabetic patients. Hyperglycemia causes elevation of reactive oxygen species (ROS) levels via mitochondrial respiratory system [3], glucose autoxidation [4], formation of advanced glycation end products (AGEs) [5], and antioxidant enzyme inactivation [6]. Elevated ROS could be extremely harmful to the major components of the cell, including nucleic acids, proteins and lipids [7]. Detoxification of elevated ROS levels is dealt with powerful antioxidant systems involving non-enzymatic [ascorbate, glutathione (GSH), carotenoids and tocopherols] and enzymatic antioxidants [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)] [8].

Crocin, one of the major components of saffron, is a carotenoid pigment and possesses the structure of crocetin di-gentiobiose ester [9]. Previous studies have demonstrated that saffron and its major ingredients (crocin, crocetin and safranal) exhibit a variety of beneficial effects including hypolipidemic, hypoglycemic, antiatherosclerotic, nephroprotective and antitumour effects in rats [10,11]. Saffron extract, crocin and safranal have also radical scavenging activities and antioxidant activity [12].

The present study was conducted to evaluate the effect of crocin on mRNA expression levels of antioxidant enzymes; SOD, CAT and GPX in the brain of the STZ induced diabetic rats.

Materials and Methods

Experimental Design

The brain tissues were obtained from our previous study [13]. Animals were procured from Inonu University, Faculty of Medicine, Experimental Research Department. Female Wistar rats (170–200 g) were housed in polypropylene cages under standard environmental conditions with a light (12 hours) and dark (12 hours) cycle, and temperature was kept at 21±2°C. Rats were fed a standard rodent pellet diet and given water *ad libitum*. The study was conducted Inonu University Experimental Animal Research Laboratory. The experimental protocol was evaluated and approved by the Inonu University Ethics Review Committee, Faculty of Medicine, Malatya, Turkey. We designed experimental groups and obtained tissue samples from our previous study as detailed below.

At the onset of the study, blood samples were extracted from the tail vein of each rat for glucose analysis using reagent strip (Accu-Check Active Glucose test strips, Roche, Germany) and a glucometer (Accu-Check Active, Roche Germany). Thirty animals were randomized in three groups containing ten animals in each group as follows;

- group 1, control (non-diabetic rats); received normal saline by gavage.
- group 2, DM (STZ-induced untreated diabetic rats); received normal saline by gavage.
- group 3, DM+Crocin (STZ-induced diabetic rats were treated with crocin); received crocin (Sigma Chemical Co., St. Louis, MO, USA.) 20 mg/kg bw/day by gavage [14]. Crocin was dissolved in normal saline.

On the first day of the study, streptozotocin (STZ;Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 0.01 M sodium citrate buffer (pH 4.5) and injected intraperitoneally (i.p.) at a single dose of 50 mg/kg body weight (bw) to DM and DM+crocin groups for induction of diabetes. Blood was taken from tail vein for the measurement of glucose levels 72 hours after the STZ injection. Rats with blood glucose levels higher than 270 mg/dL were accepted as diabetic. We found that STZ treated rats had high blood glucose levels [15]. All administrations were continued until the end of the study (for 21 days) at the same hour of the day and with a volume of 5 mL/kg bw/day. At the end of the study, all rats were killed under the xylazine (Bayer Birlesik Alman Ilac¸ Fabrikalari T.A.S., Istanbul, Turkey) and ketamine (Parke Davis, Istanbul, Turkey) anesthesia at the dose of 10 mg/kg and 50 mg/kg, respectively. The brain tissues were carefully removed to avoid damage.

Quantative PCR

The samples for laboratory investigation were stored in 1 ml of RNA-later solution at -86°C until the assays were performed. Total RNA isolation was performed from rat brain tissue with High Pure RNA Tissue Kit (Roche, No:

Table 1: Primer sequences.

Genes	Primer Sequences (Forward and reverse)	RefSeqNumber	Amplicon size
β–actin	F: 5' CTAAGGCCAACCGTGAAAAG 3' R: 5' GCCTGGATGGCTACGTACA 3'	NM_031144	79 bp
SOD	F: 5' GGTCCAGCGGATGAAGAG 3' R: 5' GGACACATTGGCCACACC 3'	NM_017050	78 bp
GPX	F: 5' GCAATCAGTTCGGACATCAG 3' R: 5' CACCGGGTCGGACATACTT 3'	NM_030826	75 bp
CAT	F: 5' AATGAAGACAACGTCACTCAGG 3' R: 5' TGTTCTCACACAGGCGTTTC 3'	NM_012520	82 bp

12033674001). Total RNA was run on 1% agarose gel and the degradation of the mRNA was checked by visualization of ribosomal bands with ethidium bromide over a UV transilluminator. The concentrations of the purified RNA were determined by a spectrophotometer (Biotek, Epoch). Transcriptor First strand cDNA Synthesis Kit (Roche, No: 04896866001) was used for reverse transcription (RT) reactions, and the manufacturer's suggested protocol was applied. Oligo (dT)-18 primer and random hexamer primers were mixed in the same reaction and used to extend all mRNA, and equal amounts of total RNA was added to each reverse transcription reaction. Quantitative PCR (qPCR) was carried out using Fast Start Essential DNA Probes Master Kit (Roche, No: 06402682001) and real time ready assays (Roche, *β-Actin* lot: 90015882, *SOD* lot: 90015884, *CAT* lot: 90015881, *GPX* lot:90015883) (Table 1) with Real Time PCR instrument (Roche LC96). The PCR mixture contained 2.5 µl of cDNA, 2 µl PCR grade water, 0.5 µl of real time ready assay mix hydrolysis probe, and primers, and 5 µl of Fast Start Essential DNA Probes Master kit. Amplification was performed at 95°C for 10 min, followed by 55 cycles of 95°C for 10 sec denaturation, 60°C for 30 sec annealing, and 72°C for 1 sec extension.

All qPCR were performed in three replicates in the same plate including the housekeeping gene. After amplifications, PCR products were run in 2% agarose gels, and single and appropriate size DNA bands were obtained for *β-Actin*, *SOD*, *CAT* and *GPX* (Figure 1). Relative mRNA expression levels of *SOD*, *CAT* and *GPX* were calculated based on *β-Actin* housekeeping gene using the $2^{-\Delta\Delta Ct}$ method [16].

Statistical analyses

Data are presented as mean and ± standard error of mean. Initially, Shapiro-Wilk test was conducted to find out if the spread of data were parametric or non-parametric. After confirming the normal distribution ($P>0.05$), data were analyzed by one-way ANOVA, which was followed by post-hoc Tamhane's T2 test using SPSS 21.0 for Windows. P values smaller than 0.05 were considered statistically significant ($P<0.05$).

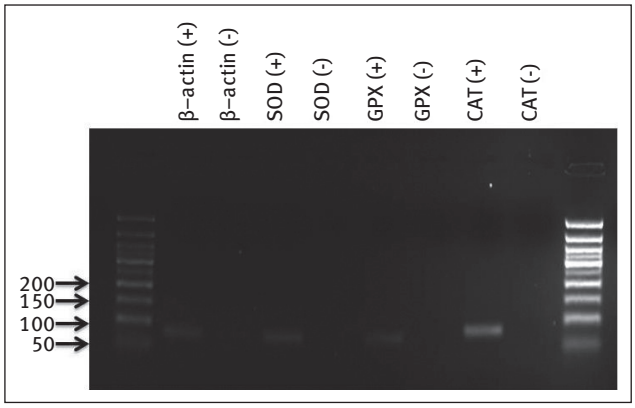

Figure 1: Agarose gel electrophoresis RT-PCR results of *β –actin*, *SOD*, *GPX* and *CAT* mRNA. The products were loaded on 2% DNA agarose gel and the size of each mRNA was determined by a DNA marker (Bioron, 50 bp, catalog no: 304007). PCR conditions and expected size of PCR products were given in "materials and methods" section. mRNA was not loaded in the negative lanes to indicate the absence of primer dimers.

Results

To observe the mRNA expression of antioxidant enzymes, SOD, CAT and GPX in the brain of the STZ-induced diabetic rats, we compared these parameters in Control, DM and DM+Crocin groups. In brief, STZ injection caused a significant increase in mRNA expression of antioxidant enzymes, SOD, CAT, and GPX when compared to control group. Crocin administration to diabetic rats significantly decreased mRNA expression of antioxidant enzymes, SOD, CAT, and GPX when compared to the DM group (Figure 2, 3, 4).

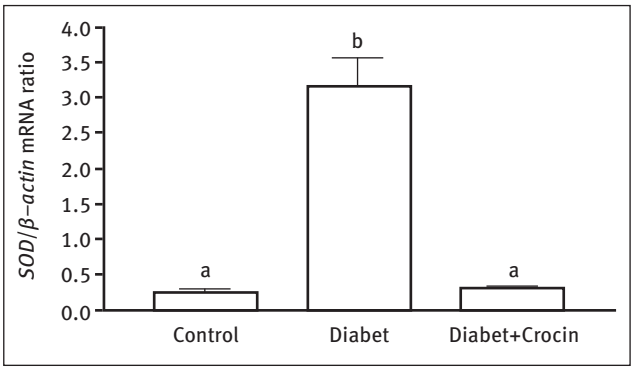

Figure 2: The ratio of brain *SOD*/*β-Actin* mRNA levels in treatment groups (Sample size for each group was n=10). The values are shown as mean and ± standard error of mean. Different letters indicate statistically significant differences between the groups (P<0.05).

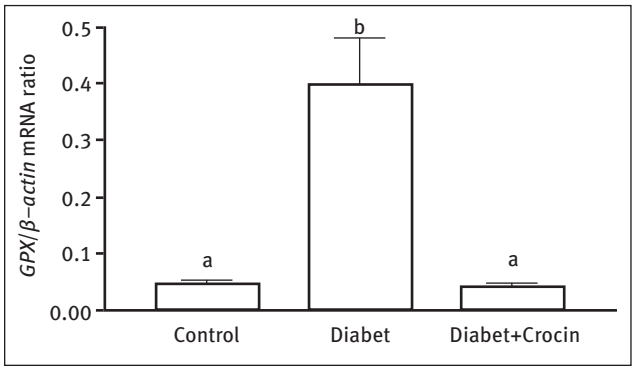

Figure 4: The ratio of brain *GPX*/*β-Actin* mRNA levels in treatment groups (Sample size for each group was n=10). The values are shown as mean and ± standard error of mean. Different letters indicate statistically significant differences between the groups (P<0.05).

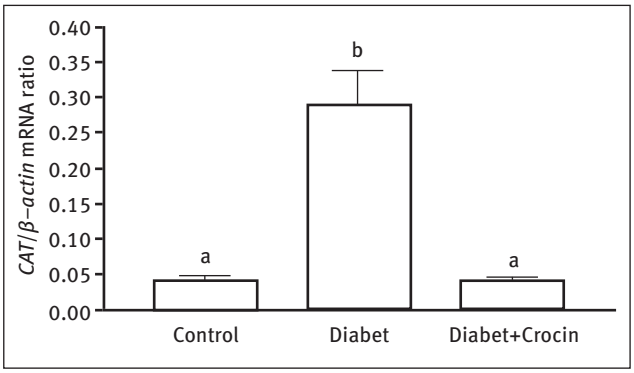

Figure 3: The ratio of brain *CAT*/*β-Actin* mRNA levels in treatment groups (Sample size for each group was n=10). The values are shown as mean and ± standard error of mean. Different letters indicate statistically significant differences between the groups (P<0.05).

Discussion

The aim of this study was to investigate the effect of crocin on mRNA expression levels of antioxidant enzymes (SOD, CAT, GPX) in the brain of STZ induced diabetic rats.

The results of the present study demonstrate that crocin treatment regulates mRNA expression of antioxidant enzymes, SOD, CAT, and GPX in the brain of the STZ-induced diabetic rats. This is the first study evaluating the effects of crocin on mRNA expression of antioxidant enzymes in the brain tissue of diabetic rats.

In the current study, we have found that STZ treatment induced diabetics and increased mRNA levels of SOD, CAT, and GPX enzymes. Previous studies also reported that mRNA levels of antioxidant enzymes were increased in diabetes mellitus [17,18]. However, certain studies provided different results based on these reports. Agardh *et al*. [19] indicated that SOD mRNA increased, but GPX mRNA did not show an increase in diabetic kidney tissue. In addition, Kishi *et al*. [20] observed that mRNA expression of SOD, CAT, and GPX did not change in experimental diabetic neuropathy. Also, Fujita *et al*. demonstrated that mRNA expression of SOD and GPx in the kidney and heart tissues increased in KKAy diabetic mice, but did not change in STZ diabetic mice [21]. These results indicated that gene expression of antioxidant enzymes in diabetic condition might differ by organ and type of diabetes mellitus.

The elevated glucose level in neurons contributes to an increase in intracellular glucose oxidation [22], which causes an increase in ROS [23]. It is well established that the central nervous system (CNS) is especially vulnerable to ROS, (for example, during high oxygen consumption of the brain for high energy requirements) and neuronal membranes are rich in polyunsaturated fatty acids, which are especially sensitive to free radical attack and could lead to elevated ROS levels. Therefore, oxidative stress could play a key role in brain damage [24].

Our results revealed an increase in mRNA expression levels of SOD, CAT, and GPX in response to STZ diabetic conditions. Some studies reported that mRNA expression levels of antioxidant enzymes were in correlation with enzymatic activity [25,26]. The change in the expression levels of these antioxidant enzyme genes might be an important results of ROS-related damage in the brain or in diabetes. The changes in antioxidant enzyme levels including SOD and CAT in neurons were consistent with those under increased oxidative stress [27]. Saffron involves several carotenoids such as crocin with high antioxidant capacity, and thus it could protect CNS neurons against oxidative stress [14,27]. Ghadrdoost *et al*. [28] stated that saffron and crocin regulated oxidative marker levels in the hippocampus. They also found that chronic stress resulted in lipid oxidation in the hippocampus, and this effect was prevented by both saffron and crocin treat-

ment. Recently, Joukar *et al.* [29] declared that saffron and crocin lead to reduction of lipid peroxidation products in some pathological conditions.

The present study demonstrated that crocin treatment of rats resulted in a decrease in mRNA levels of SOD, CAT, and GPX in the brain of STZ diabetic rats. Antioxidant and other cell redox state regulating enzyme systems including SOD, CAT and GPX enzymes provides protection against ROS in all cellular compartments [30]. The introduction of crocin acts as a free radical scavenger and an antioxidant [29] that might play as a neural protector. Several studies have shown that increases in oxidant production as well as dramatic changes in various defense system activities were closely related to alteration in gene expression of a variety of tissues from phylogenetically diverse organisms [29–31].

Conclusion

In conclusion, the present study demonstrated that crocin could modulate mRNA expression of antioxidant enzymes, SOD, CAT, and GPX, and thereby the oxidative stress in the brain of the STZ induced diabetic rats. In addition to gene expressions of antioxidant enzymes in brain tissues, in future, histopathological, immunohistochemical and biochemical analyses should be conducted to identify the effects of crocin in the treatment of diabetes.

Acknowledgement: This study was sponsored by a grant from Scientific Research Fund of Karabuk University (Grant number: KBU-BAP-14/1-KA-042).

Ethical Approval: The study was conducted in Inonu University Experimental Animal Research Laboratory. The experimental protocol was evaluated and approved by Inonu University Ethics Review Committee, Faculty of Medicine, Malatya, Turkey.

Conflict of Interest: The authors have no conflict of interest.

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