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Effect of crocin carotenoid on *BDNF* and *CREB* gene expression in brain ventral tegmental area of morphine treated rats

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ABSTRACT

Objective: To investigate the effect of crocin carotenoid on *BDNF* and *CREB* gene expression in the brain ventral tegmental area (VTA) and the serum level of BDNF in morphine-treated rats compared to control. **Methods:** In this study, 40 male Wistar rats (200-250 g) were used in 5 experimental groups: 1) non morphine treated rats (control); 2) non morphine-treated rats with 25 mg/kg crocin carotenoid (i.p., for 21 d); 3) morphine treated rats (10 mg/kg twice a day, s.c., 21 d); 4 and 5) morphine-treated rats with 12.5 and 25 mg/kg crocin carotenoid, respectively. By the end of research, *BDNF* and *CREB* expression was determined by real-time-PCR method. ELISA analysis was also applied for assessing the serum BDNF level. **Results:** The data indicated that morphine treatment could cause a significant decrease in *BDNF* and *CREB* gene expression ($P < 0.01$ and $P < 0.001$, respectively) in brain VTA as well as serum level of BDNF ($P < 0.01$) in comparison to control group. Treatment with 25 mg/kg crocin carotenoid caused a significant enhancement in *BDNF* and *CREB* gene expression ($P < 0.01$ and $P < 0.05$, respectively) and serum level of BDNF ($P < 0.01$) in morphine-treated rats in comparison to morphine-treated group. **Conclusions:** Regarding to obtained results, crocin carotenoid can inhibit unfavorable effects of morphine on the neural system to some extent through enhancing *BDNF* and *CREB* gene expression in brain VTA and serum level of BDNF.

1. Introduction

Morphine is a very important drug, which is a powerful painkiller[1]. Opiate dependence is caused by long-term consumption of these materials[2]. It has been illustrated that chronic use of opioid

drugs could cause long-term changes in brain[3]. Regarding to the definition of American Society of Addiction Medicine Association, addiction is a chronic disease, making procedures such as joy,

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reward, memory and incentives in brain[4]. The μ -opioid receptor of opiates is positioned in some areas of brain regulating feeling of pain like gray cortex, thalamus and cortex and the ventral tegmental area (VTA) and nucleus accumbens (NAc) regulating understanding of the joy and rewarding. Therefore, they could describe the reason of analgesic properties and joyful and euphoric properties created by opiates[5]. Dopaminergic neurons originated from the VTA area of neurons are sent to NAc and could be affected by rewards such as natural rewards like eating, sexual issues and several medicines. Under such conditions, controlling the GABAergic interneurons in the VTA area is removed from the dopaminergic neurons and causes neural to shoot an increased level of extracellular dopamine in the NAc and as a result, punishment process is started[6]. The main neurotrophin brain-derived neurotrophic factor (BDNF) is the main neurotrophin in the brain, which could activate intracellular signaling through binding to its receptors, which is essential to neuronal survival, neuronal differentiation and regulation of learning and memory[7]. Moreover, BDNF plays a role in transcription and translation of proteins involved in several steps of synapse development and is also an underlying factor in the pathology of neural disease like Alzheimer's disease, schizophrenia and depression[8]. In opioid-dependent individual, the serum level of BDNF is decreased[9]. Furthermore, in morphine-dependent situation, *BDNF* gene exon is transcribed in low level. In these individuals, *BDNF* gene expression in the VTA is reduced[10]. Cyclic AMP response element binding protein (CREB) is a central protein distributed in the whole body and its particular molecular mass is in average equal to 46 kDa. CREB is recognized as one of the transcription factors[11]. CREB helps control of cell growth steps such as proliferation, differentiation, survival and neural formation, learning and memory[12]. CREB regulated the expression of genes like *BDNF* and plays a key role in the mediation of response of neurotrophin[13]. As *BDNF* gene expression is regulated by CREB, decreased level of CREB could reduce BDNF level and hence, it might lead to damage of neural system due to the function of neurotrophins in the brain. Saffron contains numerous compounds such as α -protein (a water-soluble carotenoid), crocins (including crocin, dicrocin, tricrossin, picrocrocins)[14]. It has been shown that saffron or its compounds (such as crocin) have various pharmacological properties such as antioxidant and neuroprotection[15]. Saffron could decrease sedimentation and accumulation of beta-amyloid in the brain[16]. Additionally, it has been shown that crocin has learning and memory space increase properties[17]. The moment it was mentioned, BDNF is an underlying factor for neural growth and CREB could regulate the gene transcription and may play a key role in important processes such as neural survival and regulation of cognitive processes such as memory space and learning. Consequently, decrease of *BDNF* and *CREB* gene expression could pave the way for neural disorders such as depressive disorder and Alzheimer. The main objective of this

research was to investigate the effect of crocin carotenoid on *BDNF* and *CREB* gene expression in the brain VTA area and serum level of BDNF in morphine treated rats.

2. Materials and methods

2.1. Animals

In this study, 40 male Wistar rats of (200-250 g) were used. The rats were retained in separate cages and in the animal house of Rafsanjan University of Medical Sciences under the temperature of 22-24 °C and the 12-hour dark lighting period. During the study time, food and water were provided for them freely. Working with animals was done based on the approval of the Ethics Committee of Rafsanjan University of Medical Sciences (IR.Rums.REC.1395.38) based on US NIH Laboratory Animal Usage and Care Rules.

2.2. The experimental groups

In this study, 5 different experimental groups (each group including 8 rats) were examined:

1) Non morphine remedied rats; 2) non morphine-treated rats + 25 mg/kg crocin; 3) morphine remedied rats (10 mg/kg); 4 and 5) morphine-treated rats + 12.5 and 25 mg/kg crocin, respectively.

2.3. Morphine and crocin injection

The rats were treated with morphine sulfate (10 mg/kg) with an interval of 12 h for 21 d, subcutaneously (s.c.). Morphine sulfate (Temad, Tehran, Iran) was dissolved in a volume of 10 mg/1 mL physiological saline before each injection. In the crocin treated groups, the crocin was injected in the form of intraperitoneal injections (i.p.) to 12.5 and 25 mg/kg per day at the beginning of the research to the end of a period for 21 d[18]. At the end of the study period (day 21), the rats were anesthetized (50 mg/kg ketamine and 4 mg/kg Xylasein, i.p. injection) and decapitated using the guillotine. For molecular analysis, the VTA was separated from other areas as a result of coordinates of the Watson-Paksinus Brain Atlas. The blood vessel of rats was accumulated in the flask for separation of serum and analysis of the level of BDNF protein. The blood samples were centrifuged for 15 min with speed of 1 000 rpm to accumulate the blood serum. The collected serum was retained in freezer with temperatures of -20 °C for further measurements[19].

2.4. Measurement of BDNF

The BDNF level in serum was measured by ELISA method and using ZellBio Kit (Germany) and based on the instruction of the manufacturing company.

2.5. Serum glucose, triglyceride and cholesterol level analysis

The serum fasting blood sugar (FBS), cholesterol and triglyceride were examined using auto-analyzer device (KonE, Finland).

2.6. RNA extraction and complementary DNA (cDNA) synthesis

Total RNA extraction of cell was done on the tissue separated from VTA area using RNA Pars Tous Kit (Iran) according to its protocol. RNA sample was dissolved in diethylpyrocarbonate-treated water and RNA measures were determined by an APEL PD-303UV spectrophotometer (Japan). The fidelity of extracted RNA was examined by electrophoresis 1% agarose and further staining with DNA Green Viewer™ and three bands with the Gel Doc were seen from each RNA sample. cDNA was performed by using a reverse transcriptase kit (Bioneer, Korea). The cDNA was stored at -20 °C or immediately used for quantitative real-time polymerase chain reaction (Real-Time PCR). The cDNA was synthesized by Bio-Rad device produced by America Hack Company. Subsequently, RNA was converted to cDNA by reverse transcriptase enzyme, which was used as a target in the RT-PCR technique. To do so, we used to 10 µL of diethylpyrocarbonate, total RNA, oligo dT, and random hexamer primers. Then, they were incubated for 10 min at 70 °C. After this step, 10 µL of RT-mix was added into micro-tube. The mixture was put at 42 °C for 60 min for cDNA synthesis. Finally, to inactivate RT-enzymes; the mixture was incubated at 95 °C for 5 min[20].

2.7. Real-Time PCR

To perform Real-Time PCR test, by using a reverse primer and a forward primer specified to particular *BDNF* and *CREB* genes as special genes and β -*actin* gene as housekeeping gene (control), the synthesized cDNAs were determined by the referred genes and using SYBR PCR master mix (Takara) in the device, System Step One™ Real-Time PCR (ABI, USA) and the Ct of *BDNF*, *CREB* and β -*actin* genes in studying group was determined. The thermal conditions of Real-Time PCR included 95 °C for 30 min for primary denaturation, 40 cycles and each cycle including 95 °C for 5 s for the denaturation, and 60 °C for 30 min for annealing and extension. In this research, β -*actin* (housekeeping gene) was used as a control. The expression level of each gene was located in the $2^{-\Delta\Delta Ct}$ -formula as a result of the level of Ct and the Ct of control gene and the difference in the number of target gene was measured in comparison to control gene (β -*actin*)[21]. The primers in this study were designed using Beacon designer 7.8 (Biosoft, USA) and were BLAST using the software program in site (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>).

BDNF: forward GTTGCATGAAGGCTGCGCCC

BDNF: reverse CTGCCCTGGGCCCATTCACG

CREB: forward CCAAACCTAGCAGTGGGCAGT

CREB: reverse GAATGGTAGTACCCGGCTGA

β -*actin*: forward AGGGAAATCGTGCGTGAC

β -*actin*: reverse CGCTCATTGCCGATAGTG

2.8. Statistical analysis

Data are expressed as mean \pm SEM. Statistical analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, USA). To compare the obtained data from different groups, One-way ANOVA test was used. Moreover, Tukey's *post hoc* test was used following ANOVA to test significance among different groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Glucose, triglyceride and cholesterol serum level

In this study, glucose, triglyceride and cholesterol level in blood serum were measured in all experimental groups. As shown in Figure 1, the serum glucose level in rats treated with morphine was enhanced significantly compared to control rats ($P < 0.001$). Treatment with 12.5 and 25 mg/kg of crocin could reduce the glucose level (dose-dependent) in rats treated with morphine. This should be mentioned that the reduction was significant compared to the group treated with morphine ($P < 0.001$) (Figure 1). The data in this study showed that treatment of rats with morphine could lead to a significant increase in serum cholesterol level compared to control group ($P < 0.01$). Moreover, it was discovered that crocin treatment wasn't able to decrease the serum cholesterol in the group treated with morphine (Figure 2). The results obtained from this study showed that treatment of rats with morphine could cause a significant increase in serum triglyceride level compared to control group ($P < 0.01$). Significant difference has not been discovered between group treated with morphine + crocin and group treated with morphine (Figure 3).

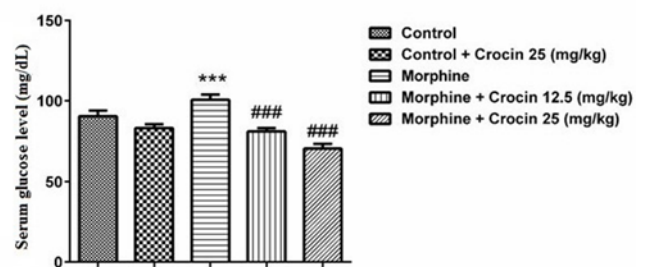


Figure 1. Serum glucose level in different experimental groups.

Data have been presented as mean \pm SEM. $n=8$ in each group; one-way ANOVA and Tukey post test are used to compare the values obtained from different experimental groups. ### $P < 0.001$, significant difference compared to morphine treated group. *** $P < 0.001$, significant difference compared to control group.

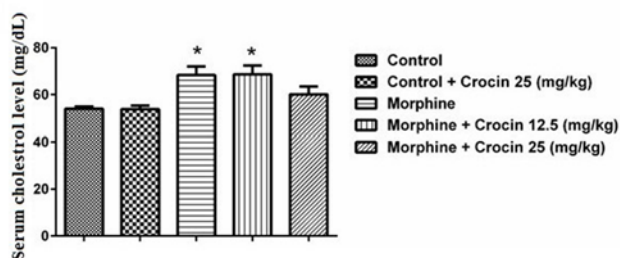


Figure 2. Serum cholesterol level in different experimental groups.

Data have been presented in form of mean \pm SEM. In each group, $n=8$; one-way ANOVA and Tukey post test are used to compare the values obtained from different experimental groups. * $P<0.01$, significant difference compared to control group.

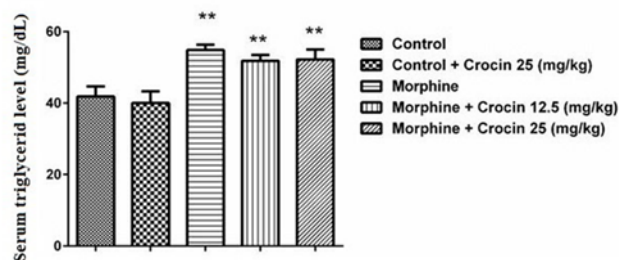


Figure 3. Serum triglyceride level in different experimental groups.

Data have been presented in form of mean \pm SEM. In each group, $n=8$; one-way ANOVA and Tukey post test are used to compare the values obtained from different experimental groups. ** $P<0.01$, significant difference compared to control group.

3.2. BDNF and CREB gene expression

BDNF and *CREB* gene expression was measured using Real-Time PCR in VTA tissue in several experimental groups. The data of these experiments showed that treatment of rats with morphine leads to significant decrease in *BDNF* and *CREB* gene expression in the VTS area of these rats in comparison to control group ($P<0.01$ and $P<0.001$ respectively). This should be mentioned that *BDNF* and *CREB* gene expression in the VTA area of rats treated with morphine + 25 mg/kg crocin was significantly higher than that of a group treated just with morphine ($P<0.01$ and $P<0.05$ respectively) (Figures 4 and 5). No significant difference was observed between the group treated with morphine + 12.5 mg/kg crocin and solely morphine-treated group (Figures 4 and 5).

3.3. BDNF serum level

The BDNF protein level was measured using ELISA method. As illustrated in Figure 6, the BDNF serum protein level in morphine-treated rats was significantly decreased in comparison to control group ($P<0.01$). The crocin administration at dose of 25 mg/kg in morphine treated rats could lead to significant enhancement in serum BDNF level in comparison to the group treated with morphine ($P<0.01$) (Figure 6).

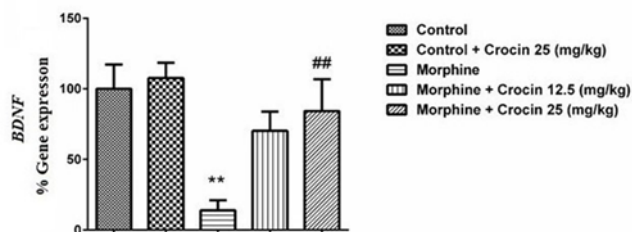


Figure 4. *BDNF* gene expression in brain VTA area in different experimental groups.

Data have been presented in form of mean \pm SEM. In each group, $n=8$; one-way ANOVA and Tukey post test are used to compare the values obtained from different experimental groups. ** $P<0.01$, significant difference compared to control group; ## $P<0.01$, significant difference compared to morphine-treated group.

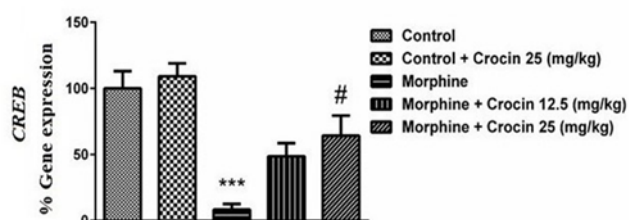


Figure 5. *CREB* gene expression in brain VTA area in different experimental groups.

Data have been presented in form of mean \pm SEM. In each group, $n=8$; one-way ANOVA and Tukey post test are used to compare the values obtained from different experimental groups. *** $P<0.001$, significant difference compared to control group; # $P<0.05$, significant difference compared to morphine-treated group.

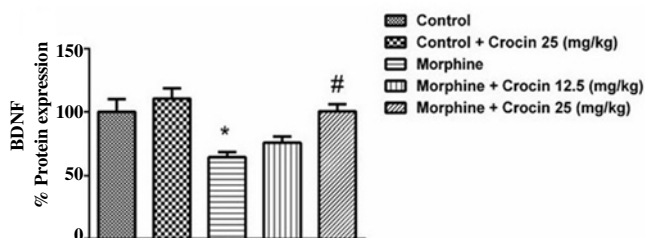


Figure 6. BDNF serum protein level in different experimental groups.

Data have been presented in form of mean \pm SEM. In each group, $n=8$; one-way ANOVA and Tukey post test are used to compare the values obtained from different experimental groups. * $P<0.01$, significant difference compared to control group; # $P<0.01$, significant difference compared to morphine-treated group.

4. Discussion

In this research, the effect of crocin on *BDNF* and *CREB* genes expression as well as BDNF serum level was studied in morphine-treated rats. Regarding to the obtained outcomes, treatment with morphine could lead to significant decrease of *BDNF* and *CREB* gene expression in the brain VTA area and decrease of BDNF serum level in comparison to control rats. Furthermore, the data

showed that treatment with crocin at dose of 25 mg/kg could cause significant enhancement in *BDNF* and *CREB* gene expression in the brain VTA area as well as serum BDNF level in rats treated with morphine. It has been specified that there are a lot of μ -opioid receptor in the brain VTA area and their activation related to the rewarding effects of morphine[22]. In addition, previous researches have demonstrated that the addictive behaviors caused by morphine are depended on the activation of dopaminergic neurons in the brain VTA area. Therefore, reward response produced by activation of VTA-dopaminergic neurons by morphine could be one of the key factors of dependence on addictive substances[23,24]. BDNF is recognized as a factor for brain growth, neural reconstruction and synapse plasticity[25]. BDNF receptors are plentifully available in the VTA-dopaminergic neurons[26]. BDNF might lead to survival and protection of dopamine neurons in the brain[27,28]. In this field, it has been revealed that significant decrease of BDNF in the substantia nigra brain of Parkinson patients may lead to degeneration and destruction of these dopaminergic neurons[29]. Different studies have affirmed the effect of BDNF in learning, memory, identification, perception and regulation of various types of feelings. Moreover, BDNF disruption that can involve in many cognitive disorders such as schizophrenia, huntington, autism, depression, addiction and anxiety disorders has been studied[30,31]. CREB is transcription factor activated in response to signaling routes such as calcium-calmodulin, growth factor and cytokines. Phosphorylation of CREB protein at ser133 by protein kinase A, MAP kinase and extracellular signal-regulated kinase could activate several gene transcription[32]. One of the target genes of CREB is *BDNF*, which is responsible for regulating their gene expression[33]. Deactivation or decrease of CREB could reduce *BDNF* gene expression. In addition, the BDNF-TrkB signaling route could lead to activation of CREB through activating extracellular signal-regulated kinase and hence, the two factors could form a positive response loop[34]. The review conducted by Koo *et al.* on morphine-depended rats showed that, the *BDNF* gene expression level is significantly reduced in VTA area. Furthermore, they discovered that the activity of CREB decreases in morphine-depended rats[35]. Yang *et al.* showed that chronic exposure of rat to morphine could decrease CREB phosphorylation level in the hippocampus area[36]. Zhou *et al.* demonstrated that morphine injection to rats can decrease CREB level in NAc[37]. According to the outcomes of present research, chronic morphine treatment could cause significant reduction of *CREB* gene expression in the brain VTA area of male Wistar rats. Zhang *et al.* compared heroin-dependent people (an opioid derivative) with normal people and found that in heroin-dependent individuals, serum BDNF level was decreased compared to controls ($P < 0.01$)[38]. The research conducted by Angelucci *et al.* demonstrated that in heroin-dependent individuals, serum BDNF level is decreased and due to the function of the proteins, and decrease of its level in these individuals could raise the risk of psychosis disease[39]. The research performed by

Ghodrati *et al.* on rats under treatment with morphine demonstrated enhanced level of anxiety, memory dysfunction and reduced BDNF level in hippocampus[40]. Nunes *et al.* demonstrated that exposing rat infants to morphine could decrease the BDNF level in their cerebral cortex[41]. According to Lee *et al.*, *BDNF* gene expression is reduced in hippocampus of morphine-depended rats[42]. Research performed by Chen *et al.* also showed that in rats exposed to chronic use of morphine, serum level and the BDNF protein expression were decreased in NAc area[43]. Koo *et al.* demonstrated that in morphine-depended individuals, decrease of expression and signaling of TrkB-BDNF could cause enhanced bonus process induced by morphine consumption. This seems that this function is caused by controlling GABA receptor in the NAc area[44]. Moreover, the present research discovered that morphine-dependence could decrease *BDNF* gene expression in VTA area and its serum level in male rats significantly. Decreased serum and cerebral level of BDNF may demonstrate special amount of neural degeneration caused by long-term drug abuse in the body. Regarding to these results, using medicines may have an incremental impact on neurotrophins and could improve the effects of drugs on neural cells, which may be useful in the treatment of addiction to opioids. Vahdati *et al.* has researched the effect of saffron crocin on *BDNF* and *CREB* gene expression and found that crocin could enhance the levels of the two types of proteins in the hippocampus area of rats[18]. Ghasemi *et al.* studied the anti-depression effects of saffron (main herbal source of crocin) on rats and discovered that saffron could raise the levels of BDNF and CREB in hippocampus significantly[45]. In the study of Liu *et al.*, chronic morphine administration induced upregulation of BDNF. Inhibiting BDNF effectively delayed the generation of MIH and tolerance. The upregulation of BDNF induced by morphine was significantly suppressed by inhibiting Shh signaling. Inhibiting BDNF significantly suppressed smoothened agonist-induced hyperalgesia[46]. In the study of Razavi *et al.*, in long term crocin treatment, a slight increase was observed in protein level of P-CREB, but it was not significant. It is concluded that antidepressant activity of crocin might be partially mediated by CREB[47]. In the present study, we showed that crocin could significantly increase *BDNF* and *CREB* gene expression in the brain VTA of morphine-treated rats. Additionally, administration of crocin could elevate the serum BDNF level in morphine-treated animals significantly.

Altogether the results obtained from this study showed that chronic treatment of rats exposed to morphine could decrease expression of *BDNF* and *CREB* genes in the VTA area as well as serum BDNF level. Furthermore, the results obtained from this study showed that crocin administration with morphine could increase *BDNF* and *CREB* gene expression in the VTA area as well as serum BDNF level. The reduction of BDNF protein expression could lead to underlying difficulties and may lead to other species in the neural system. Hence, crocin as a complementary factor could be considered as remedying of morphine administration, side effects

in opioid depended patients or the people treated with morphine for chronic period.

Conflict of interest statement

The authors of present study declared there is no conflict of interest.

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