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Original Article

Evaluating the Effect of Crocin on NRAS, KRAS, c-FOS, and c-JUN Genes Expressions in Lung Tissue of Cadmium-Treated Rats

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Abstract

Background: Cadmium (Cd) is an environmental pollutant known to be toxic to lung tissue. Crocin is an active phytochemical of saffron with antioxidant properties. Because of this characteristic, it is hypothesized that crocin can reduce the harmful effects of toxic chemicals. The current study aimed to investigate the crocin intervention on the expression of NRAS, KRAS, c-FOS, and c-JUN genes in the lung tissues of Cd-treated rats.

Methods: In this study, 40 Wistar rats (180±30 g) were randomly divided into four groups: the control group, received food and water; the crocin-treated group, received 15 mg/kg crocin orally; the Cd-treated group, received 20 mg/kg Cd by gastric gavage; and the complex group, treated by crocin and cadmium with previously mentioned concentrations. After eight weeks daily administered, the rats were euthanized, and their lungs were extracted to assess NRAS, KRAS, c-FOS, and c-JUN gene expressions by Real-Time PCR. Data were computed by GraphPad Prism (v.8). One-way ANOVA test was used as statistical analysis, and $P<0.05$ was considered statistically significant.

Results: The results revealed that the Cd consumption raised the expression of all four genes in lung tissue (c-JUN, KRAS $P<0.001$, NRAS $P<0.01$, and c-FOS $P<0.05$). Crocin significantly reduced the expression of c-JUN ($P<0.0001$), c-FOS, and NRAS ($P<0.05$) genes.

Conclusion: The data obtained from the current study indicated that crocin could reduce the expression of c-FOS, c-JUN, and NRAS as vital players in cell proliferation.

Keywords: Lung Damage, Cadmium, Crocin, Cell Proliferation, NRAS, KRAS, c-FOS, c-JUN.

1. Introduction

Annually millions of new cancer cases are diagnosed worldwide. Both genetics and environmental factors influence the progression of cancer. It has been estimated that around 50 percent of cancers are caused by environmental factors and lifestyle [1]. The cancer

progression is a multi-step process which can lead to malignancy several years after exposure to a carcinogen [2]. Lung cancer is the fourth most prevalent malignancy worldwide and stands as the most frequent cause of cancer death, according to the 2020 GLOBCAN report. In Asia, lung cancer incidence, prevalence, and mortality in both men and women are higher than in global records [3]. Lung cancer treatment is a complicated process, and the survival is low. Genetic variation and environmental pollution are responsible for lung cancer progression [4].

Some of environmental risk factors attributing to lung malignancy are ultra-violet, tobacco (in both active and passive smokers), asbestos, chemicals (cadmium, cobalt, and the other chemical found in air pollution) [5]. Cadmium (Cd) is a trace heavy metal [6] and is a recognized human carcinogen according to International Agency for Research on Cancer (IARC) as a group I carcinogen. Cd has a long half-life in the body and a low excretion rate, resulting in Cd accumulation in the body [7]. Cd can induce the cancerogenesis process via several mechanisms [8]. Oxidative stress possibly plays a vital role in Cd carcinogenic property and causes dysregulation of gene expression, blocking DNA repair and repressing apoptosis [9]. Chronic Cd inhalation has been proven toxic to the lung. The international efforts to reduce air Cd pollution and decrease smoking were successful enough. Hence, nowadays, the Cd oral consumption is the predominant source of Cd toxification. Cd oral exposure can cause its accumulation in the lungs and leads to the obstructive lung diseases, bronchitis, and lung cancer, as well [10].

The mitogen-activated protein kinase (MAPK) pathway transmits extracellular signals to intracellular cognate molecules, which regulates cell destination. KRAS and NRAS, members of the RAS subfamily, are transmembrane GTP- binding proteins. They are the initial relay point in the given pathway [11]. *KRAS* mutations occur in a significant proportion of non-small cell lung cancer (NSCLC) cases and are associated with poor prognosis. It has been revealed that the expression of *KRAS* raised in most types of lung cancer [12]. Unlike *KRAS* prevalent mutations, *NRAS* mutations reveal particular therapeutic characteristics which change their expression pattern [11]. The MAPK pathway can induce the transcription of activated protein 1 (AP-1) subunits. AP-1 is a significant player in cell migration, cell proliferation, apoptosis, metastasis, and inflammatory reactions. The early response transcription factor AP-1 consists of c-FOS and c-JUN. c-FOS molecule possesses a short half-time; however, its expression raised rapidly by induction of a stimulant [13]. C-Jun is a proto-oncogene which actively involved in various cellular processes through interaction with different signal transduction pathways [13].

Accumulating evidence exhibits the role of Cd in the induction of MAPK signaling pathway [14] and oxidative sensitive transcription factor AP-1 pathway [15]. Therefore, trying to find a chemopreventive component is of interest to decrease the toxic effects of Cd. In this regard, herbal components are appealing. Phytochemicals are are naturally existing in plants and are responsible for characteristics like color and smell [16]. Studies revealed that phytochemicals could regulate the cell cycle through various signaling pathways, mostly ceasing tumorogenesis and cancerogenesis progression [17–19].

Crocus sativus L. or Saffron is a plant of the order Asparagales and the family *Iridacae* [20]. Phytochemical analysis of saffron introduced crocin as one of its main chemical components. Crocin is a

carotenoid compound responsible for saffron's color. Saffron extract and its phytocomponents, including crocin, have antioxidant, anticancer, and anti-inflammatory properties and have been extensively examined in vitro and in vivo. Crocin is of high significance for its abilities in cancer cell suppression, inducing apoptosis in malignant cells [21]. Herbal active ingredients can be used as a stimulant or inhibitor in the treatment of disorders and diseases [22].

Previous studies revealed the inhibitory effects of the crocin on oxidant tissue damage. The current study examined crocin effects on N-RAS, K-RAS, c-FOS, and c-JUN expressions in rats' cadmium-damaged lung tissue.

2. Materials and Methods

2.1. Animal treatment

Forty male Wistar rats aged eight-week-old (180±30 g) were provided from Pasteur Institute. The rats were maintained under the standard environmental conditions (21-25 °C, 50±5% of humidity, and 12 h light: 12 h dark photoperiod) with access to food and water ad libitum. The experiment was carried out based on the guidelines of the Principle of Laboratory Animal Care (NIH publication n. 86-23, revised 1985) and certified by Central Tehran Branch- Islamic Azad University in 2017 (Certificate No. 10130553962002).

After seven-day acclimatization to the laboratory, they were randomized into four experimental groups (n=10). The Cd (Azmiran Company, Germany) was dissolved in distilled water. Crocin (Sigma-Aldrich, Germany) was dissolved in distilled water. The treatments were introduced once daily for eight consecutive weeks as follow: control group (standard dietary), crocin group (20 mg/kg), cadmium group (15 mg/kg), complex group (crocin and cadmium together, the doses were chosen as the two latter groups) (the chemicals were administered through gavage). The doses were selected according to the previous study [23, 24].

2.2. Gene expression analysis

After 8-weak treatment, all animals were euthanized by narcotic overdose. The lungs were removed and transferred to RNA Later (Sigma-Aldrich, Germany) and stored at -80 °C.

Total RNA of lung tissue was extracted by RiboEx (GENE ALL, South Korea) according to the manufacturer's instructions. The quantity and quality of extracted RNA were measured by spectrophotometry (NanoDrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA) and 1.5% agarose gel electrophoresis. Samples with 260/280 ratio of 1.8-2 were used for complementary DNA (cDNA) synthesis. The reverse transcription was done by PrimScript RT Reagent Kit (Takara Corporation, Japan). Primers were designed by Oligo 7 software, and their sensitivity was assessed by the NCBI site (http://www.ncbi.nlm.nih.gov/blast) (Table 1). GAPDH gene was chosen as the internal control.

The synthesized cDNAs were used as a template in real-time PCR reaction by Rotor-Gene 6000 (Corbett Research, Australia). The amplification condition consisted of an initial denaturation at 95 °C for 15 minutes, followed by denaturation at 95 °C for 20 seconds, annealing at 61 °C for 20 seconds, and extension at 72 °C for 20 seconds. The process repeated for 40 cycles, and the final extension was at 72 °C for 5 minutes.

Table1. Nucleotide sequences of primers used for real-time PCR

Primer	sequence	Tm (°C)	Product size (bp)
NRAS-F	5'- AAGCGCGTGAAAGACTCTGA-3'	60	247
NRAS-R	5'- CCATACAGCCTTGAGTGCCA -3'		
KRAS-F	5'- GAGAACTGGGGAGGGCTTTC -3'	60	180
KRAS-R	5'- TCCTGAGCCTGTTTCGTGTC-3'		
c-FOS-F	5′-ACTCCCAGCTGCACTACCTA-3′	60	171
c-FOS-R	5′-CCTGCCTTCTCTGACTGCTC-3′		
c-JUN-F	5′-GTCTCAGGAGCGGATCAAGG-3′	60	172
c-JUN-R	5′-CACCTGTTCCCTGAGCATGT-3′		
GAPDH-F	5'-ATCACTGCCACTCAGAAGAC -3'	60	179
GAPDH-R	5'-ACATTGGGGGTAGGAACAC -3'		

2.3. Statistical analysis

All the tests were done for three times, and the results were reported as mean± standard deviation (SD). The gene expression was obtained in comparison to the internal control GAPDH according to $2^{-\Delta\Delta Ct}$ formula. One-way ANOVA and post hoc Tukey tests were used to evaluate the differences. GraphPad Prism v.8 was used to perform statistical analysis. A p-value less than 0.05 was considered statistically significant.

3. Results

The results of quantitative mRNA level analysis are presented in Figure 1 and Figure 2. The expression of NRAS ($p<0.001$), KRAS ($p<0.01$), c-JUN ($p<0.001$), and c-FOS ($p<0.05$) were significantly increased due to the Cd. The expression of these genes in crocin-treated animals demonstrated different reactions. c-JUN and KRAS increased in the crocin-treated group; however, only the rise in c-Jun expression was statistically significant ($P<0.05$). c-FOS and NRAS mRNA expressions were reduced due to the crocin consumption, although only the alleviation of c-Fos was statistically significant ($p<0.01$).

The expression of all examined genes was reduced in the complex group (crocin and Cd).The simultaneous consumption of crocin and Cd was most effective on c-Jun expression ($p<0.001$).

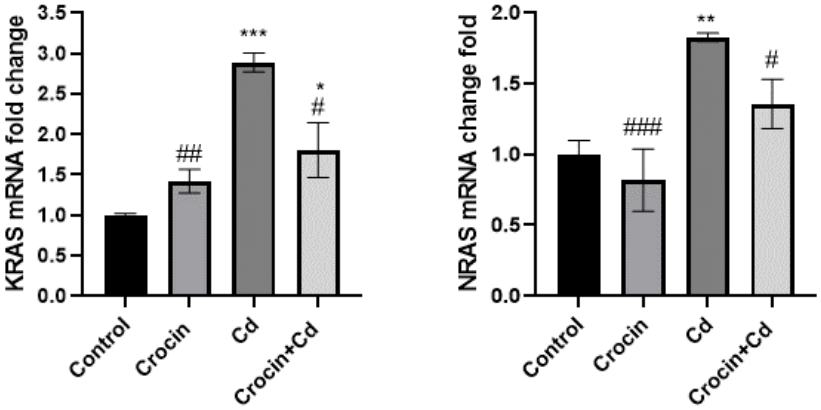

Figure 1. The relative expression of KRas and NRas genes in 4 different treated groups.
*: $p<0.05$, compared with control group; **: $p<0.01$, compared with control group;
***:$p<0.001$, compared with control group.
#: $p<0.05$, compared with cadmium treated group; ###: $p<0.001$, compared with cadmium treated group.

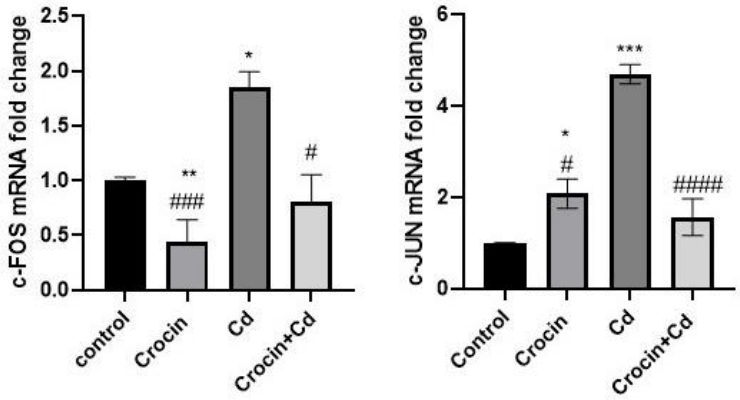

Figure 2. The relative expression of c-FOS and c-JUN genes in 4 different treated groups. *: p<0.05, compared with control group; **: p<0.01, compared with control group; ***:p<0.001, compared with control group.
#: p<0.05, compared with cadmium treated group; ###: p<0.001, compared with cadmium treated group;####: p<0.0001, compared with cadmium treated group.

4. Discussion

Crocin is a caretoneid of *Crocus sativus*. It seems that the therapeutic properties of crocin are related to its antioxidant activity [25]. Studies revealed that crocin is capable of regulating cellular amplification, differentiation, and protection of normal cellular function through inducing apoptosis and anti-inflammatory response [21, 26, 27]. Avoiding carcinogenesis is impossible due to the genetic and epigenetic changes in tumor suppressor genes and proto-oncogenes during lifetime. In addition, exogenous carcinogens in the environment increase more concerns [28]. Hence, more attempts are needed to explore the preventive therapy to overcome this obstacle.

Cadmium is listed as a carcinogen substance by World Health Organization [3]. Industrial and agricultural activities have been resulted in soil and water contamination by Cd. Then, the plants uptake Cd and store it in crops. Subsequently, humans will be exposed to Cd in their diet by consuming contaminated vegetables. Systemic Cd exposure targets several organs, including the lung and ends to lung cancer [10, 29]. It has been hypothesized that the adverse effects of Cd are mainly induced by reactive oxygen species (ROS) or inflammation [29].

Studies showed that the RAS family members' over-expression effectively disrupts the relation between cellular pro-proliferation downstream signaling and growth factor receptors. Upregulation of each RAS family gene is associated with a poor prognosis [30].

AP-1 is a transcription factor consisting of c-JUN and c-FOS, and further it plays a crucial role in cell proliferation and inflammation. The upregulation of c-JUN and c-FOS genes transforms cells and promotes tumorigenesis [31]. Regarding the role of Cd in the induction of lung cancer [9, 10] and the role of NRAS, KRAS, c-FOS, and c- JUN genes in cell transformation and tumorigenesis promotion [31], this study evaluates the crocin effect on mRNA levels of the mentioned genes in animals treated by Cd. The results revealed that crocin treatment influenced the expression of the genes. Crocin reduced the mRNA expression of KRAS, NRAS, c-FOS, and c-JUN (1.6, 1.34, 2.31,

2.99, respectively) compared with Cd-treated group, which were statistically significant.

Until recently, various studies have been carried out on the efficiency of crocin on different cancers; which had similar results. For instance, Ashrafi et al. examined the effect of crocin on breast cancer and indicated that crocin suppressed the cyclin D1 and $P21^{Cip1}$ expression. They concluded that the tumor suppressor effect of crocin resulted from cell cycle inhibition [32]. Bakhshi *et al.* demonstrated that crocin intervention could reduce the metastasis rate of C57BL/6 in mice by 85%. In their study, crocin inhibited KRAS expression in treated groups [33]. In our study, the expression of KRAS decreased in crocin-Cd group compared to Cd group. c-FOS overexpression is a marker of oxidation. Hadipour et al. study on amyloid beta-induced memory deficits in hippocamp suggested the neuroprotection of crocin by reducing c-FOS [34]. Similar to our result, the crocin treatment downregulated c-FOS. The reduction of c-FOS expression has been previously reported by Shi *et al.* and Fu *et al.* [35, 36]. According to the obtained results in the current study, it can be concluded that the crocin can attenuate the proliferation signaling pathways whose regulation was disturbed because of Cd exposure.

It is better to examine the downstream genes of these given genes. Likewise, it is important to check the expression of c-JUN NH2-terminal kinase (JNK), a linker of MAPK to AP-1. In addition, it is better to assess the lung tissue state histopathologically when gene expression is evaluated.

5. Conclusion

In conclusion, results obtained in this study revaled that crocin as a biocomponent of saffron could compensate for the adverse effects of Cd on NRAS, KRAS, c-FOS, and c-JUN expression in mRNA level. All genes expression in the Cd-crocin-treated group was decreased around their regular expression in the control group. It can be assumed that crocin can be helpful as a chemopreventive agent among those who expose chronically to Cd.

Authors' contributions

Nastaran Asghari Moghaddam and Solmaz Shahla designed this study. Farzaneh Salmanzadeh Mehmandust Olia and Marziyeh Khandan obtained and analyzed the data. Solmaz SHahla, Farzaneh Salmanzadeh Mehmandust Olia and Marziyeh Khandan proceeded to the quality control and the manuscript drafting. Nastaran Asghari Moghaddam revised the final version.

Consent for publications

All authors agree to have read the manuscript and authorize the publication to the final version of the manuscript.

Conflict declaration

The authors declare that there is no conflict.

Conflict of interest

None of the authors have any conflict of interest to declare.

Availability of data and material

Data are available on request from the authors.

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Ethics approval and consent to participate

The ethical approval was certified by Central Tehran Branch-Islamic Azad

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