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The Protective Effect of Crocin on Rat Bone Marrow Mesenchymal Stem Cells Exposed to Aluminum Chloride as an Endocrine Disruptor

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Abstract

Background: Mesenchymal Stem Cells (MSCs) have the ability to self-renew and proliferate which gives them healing properties in various tissues. Aluminium chloride ($AlCl_3$) is a chemical compound with harmful effects on health; oxidative stress caused by Aluminium has been reported previously. Crocin, a major component of *Crocus sativus* (saffron), has antioxidant properties and has shown therapeutic potential. Researchers have been looking for ways to reduce the harmful effects of $AlCl_3$.

Methods: To investigate whether crocin can reduce $AlCl_3$ cytotoxicity, rat Bone Marrow Mesenchymal Stem Cells (BM-MSCs) were isolated, cultured and divided into four experimental groups. The first group was the control, which was untreated cells. The second and third groups were treated with crocin (50, 100, 250, 500 μM) and $AlCl_3$ (20, 25, 30 *mM*) for 24 *hr*. The fourth group was pre-treated with crocin (250, 500 μM) for 24 *hr* and then treated with $AlCl_3$ (20 *mM*) overnight. Cytotoxicity was assessed using the MTT assay. Mineralization was evaluated by alizarin red staining. Sox-2 and E-cadherin expression were measured using real-time PCR.

Results: The results showed that $AlCl_3$ caused cytotoxicity on BM-MSCs and decreased the mRNA expression of Sox-2 and E-cadherin, which are important for the maintenance of self-renewal and proliferation of BM-MSCs. In contrast, crocin protected the self-renewal characteristic of BM-MSCs by increasing Sox-2 expression and also preserved the proliferative effects on BM-MSCs by upregulating E-cadherin expression (***$p \leq 0.001$).

Conclusion: Overall, the study suggests that crocin can protect BM-MSCs from $AlCl_3$-induced cytotoxicity by upregulate Sox-2 expression and E-cadherin expression. This suggests that crocin may be a potential therapeutic agent for the treatment of $AlCl_3$-induced toxicity.

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Introduction

During the past decade, several studies have investigated the effects of natural compounds on the treatment of human diseases such as osteoporosis. Osteoporosis is a type of bone disorder characterized by decreased bone deposition and increased bone resorption. Among these natural compounds, flavonoids act to inhibit bone loss by reducing oxidative stress and promoting osteoblast differentiation [1-3]. Saffron is a spice that has been used in traditional medicine for centuries. The active ingredients of saffron have demonstrated a variety of beneficial effects, including anti-proliferative, anti-tumor, anti-inflammatory, anti-oxidant, anti-apoptotic, and osteo-protective effects [4,5].

Crocin is a glycated form of saffron and is a unique

water-soluble carotenoid found in saffron. The osteoprotective effects of crocin are thought to be caused by its antioxidative properties. Crocin is a valuable agent against oxidative stress, which can be palliative for the prevention of osteoporosis [6].

Some studies have shown that crocin can be effective in controlling osteoporosis by increasing the number of T cells (Tregs), increasing the level of anti-osteoclastogenic cytokines, and reducing the number of osteoclastogenic Th17 cells [7]. Crocin also uses various intracellular pathways, including PI3K/AKT and Wnt/β-catenin signaling pathway [8]. Aluminium (Al) as an endocrine disruptor is a toxic agent that is widely present in water, food additives, therapeutic treatments and the environment. Endocrine disruptors are substances that can interfere with various bodily functions, such as fertility, immune function bone development [9]. Al can be excreted through urination, but the remaining Al can accumulate in various tissues such as kidneys, liver, heart, blood, brain and bones. Exposure to Al can damage cells and tissues and lead to various diseases such as Alzheimer, osteomalacia and bone disorders [10].

Mesenchymal Stem Cells (MSCs) are present in various tissues, such as adipose tissue, peripheral blood, umbilical cord blood, lung, and bone marrow. MSCs have the ability to differentiate into different cell types, such as osteocyte, chondrocyte, adipocyte, and other lineage [11]. MSCs have some important characteristics such as self-renewal and differentiation, which make these cells as a valuable source for healing and inhibiting the progression of diseases such as osteoporosis. These characteristics also make them a promising candidate for regenerative medicine in osteo-related diseases [12].

E-cadherin plays an important role in cell-cell adhesions, which is necessary for the maintenance of cells and tissues integrity [13]. Recently, it has been demonstrated that E-cadherin acts as a regulator of pluripotency and self-renewal signaling pathways in stem cells [14]. Morphological changes resulting from the interruption of E-cadherin expression can influence cell proliferation and differentiation, which is associated with β-catenin and Wnt signaling pathway [15]. One of the modulators of Wnt/β-catenin signaling in development and disease is Sox transcription factors. SOX2 is a core gene that forms a key network essential for the pluripotency and plays a distinct role in maintaining the self-renewal capacity and multipotency of MSCs [16]. Therefore, in this study, we investigated cytotoxicity of $AlCl_3$ (Aluminium chloride) and the protective effects of crocin against $AlCl_3$-induced cytotoxicity in rat Bone Marrow Mesenchymal Stem Cells (BM-MSCs) *in vitro*.

Materials and Methods

Rat BM-MSCs isolation and culture

All protocols used in this study were approved by the ethics committee of Kharazmi University (Ethical code: (IR.KHU.REC.1399.013). In this study, male Wistar rats (100-120 *g*) were euthanized with intraperitoneal injection of ketamine (360 *mg/kg*) and xylazine (30 *mg/kg*) followed by cervical dislocation. The lumber region to toes were shaved, skin was incised, and the tibia and femur of both legs were exteriorized as described earlier [17]. The femur and tibia of both legs were placed in petri dish containing PBS. The metaphysis region of both bones was cut, then a needle inserted into the medullary cavity of bone marrow and flushed out using DMEM (Dulbecco's Modified Eagle Medium) into a 15 *ml* centrifuge tube. The suspension was then centrifuged for 5 *min* at 2000 *rpm* to concentrate the cells. The supernatant was decanted and 5 *ml* of complete media consisting of 85% DMEM (Bioidea, Iran), 10% Fetal Bovine Serum (FBS, Bio-idea, Iran), and Penicillin/Streptomycin (100 *U/ml*) (Bioidea, Iran) was added to the tube and pipetted. After 72 *hr* of seeding, non-adherent cells removed by decanting the media. Every 3 or 4 days, the media was replaced until the flask achieved 80-90% confluency. Then, Trypsin-EDTA solution (0.25% Trypsin and 1mM EDTA) was added to passage the flasks. The MSCs were incubated at 37ºC in a humidified atmosphere of 5% CO_2 incubator. To be applied in this experiment, BM-MSCs were used at the 3rd-4th passages. Then, four experimental groups were considered. Untreated cells (control) were considered as the first group. In the second and third groups, BM-MSC were treated with various concentrations of crocin or $AlCl_3$ for 24 *hr*. In fourth group, the cells were pre-treated with various concentrations of crocin for 24 *hr*, then treated with $AlCl_3$ at IC50 concentration.

Cell cytotoxicity assay

To determine the non-toxic concentration of crocin (Puyesh Darou Sina, Iran) and the IC50 concentration of $AlCl_3$, the MTT assay was performed. In brief, BM-MSCs were plated in 96-well plates at a density of 5×10^3 cell/well 24 *hr* ahead of treatment. Untreated MSCs were considered as control. BM-MSC were treated with various concentrations of crocin (50, 100, 250, and 500 μM), $AlCl_3$ (20, 25, and 30 *mM*) or pretreated with crocin (50, 100, 250 and 500 μM) and then treated with $AlCl_3$ (20 *mM*) for 24 *hr*, in the experimental groups 2 to 4. Each treatment was repeated at least three times. After 24 *hr* of treatment, MTT solution (0.5 *mg/ml*) (Sigma, USA) was added to the wells for 3 *hr* at 37ºC. The medium was the aspirated, and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany). Finally, the absorbance was read at 570 *nm* using iMark™ Microplate Absorbance Reader (Bio-Rad, USA).

The effect of crocin on mineralization of BM-MSCs exposed to $AlCl_3$

To evaluate protective effect of crocin on rat BM-MSCs exposed to $AlCl_3$, the mineralization of BM-MSCs was investigated. In this assay, BM-MSCs (passage 3) were cultured at 2×10^5 in 6-well plates. The

cells were pretreated with crocin (50, 100, 250, and 500 *µM*) and then subjected to $AlCl_3$ (IC50 value: 20 *mM*) for 24 *hr*. The treated and untreated BM-MSCs were also cultured in osteo-differentiation medium containing DMEM supplemented with 10% FBS, 50 *µM* ascorbate acid, 10 *mM* β-glycerol phosphate and 10 *nM* dexamethasone incubated for 21 days.

After the treatment period, the cells were rinsed twice with PBS and fixed in 70% ethanol for 20 *min*. The calcium deposits were then determined by Alizarin red staining (5 *mg/ml* in PBS; pH=4.1-4.3). Then, the calcium deposits were evaluated under inverted microscopy (Olympus, Japan).

RNA extraction, cDNA synthesis, and Real-time PCR

In this study, the effect of crocin pre-treatment (250, 500 *µM*) on BM-MSCs exposed to $AlCl_3$ (20 *mM*) on the transcription level of Sox-2 and E-cadherin of BM-MCSs was evaluated by real-time PCR. First BM-MCSs (2×10^6) were seeded in T25 flasks overnight. Then, the cells were exposed to crocin (250, 500 *µM*) for 24 *hr*. After pre-treatment with crocin, BM-MSCs were treated with $AlCl_3$ (20 *mM*) which has a 50% lethal concentration (IC_{50}) for overnight. Total RNA was extracted from untreated and treated cells using total RNA extraction kit (Parstous Biotechnology, Iran) according to the manufacturer's instructions. Then, the concentration of RNA was determined using a Nano-Drop (Thermo Scientific, USA) and cDNA was synthetized from extracted mRNA for each sample using an easy cDNA synthesis kit (Parstous biotechnology, Iran) with the aid of oligo dT primers. Finally, to compare the expression of target genes in the treated groups with the control, real-time PCR was performed using SYBER Green PCR master mix (Takara, Japan) in a Corbett research rotor-gene RG6000 real-time rotary PCR (Corbett, Australia). The results were normalized to the expression of the housekeeping gene, which in this study was *GAPDH*. The specific forward (F) and reverse (R) primers (5′-3′) sequences for real-time PCR were listed in table 1. Finally, the data were analyzed using the ΔΔCt method [18].

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by post hoc comparisons. One-way ANOVA was used to compare more than two groups. The differences between groups were considered statistically significant at $p<0.05$*, $p<0.01$**, and $p<0.001$***. The SPSS software 22.0 was used for statistical analyses. Results are expressed as the Mean± SEM.

Results

In vitro expansion of rat bone marrow MSCs

The isolation and *in vitro* expansion of BM-MSCs were performed. The characterization of rat BM-MSCs was reported in our previous study [17]. The proliferative state of BM-MSCs in culture medium containing DMEM with 10% FBS prior to sub-culturing was observed using an inverted microscope (Figure 1).

Effect of crocin on $AlCl_3$-induced cytotoxicity in BM-MSCs

In the current investigation, the MTT assay was performed to determine the proper concentration of crocin that can reduce cytotoxic effects of $AlCl_3$ on BM-MSCs and cause a significant decrease in cell death compared to the control (untreated cells). The IC_{50} concentration of $AlCl_3$ was also determined. The obtained data revealed that the cell viability of the BM-MSCs was decreased when cells were exposed to $AlCl_3$ at concentrations of 20 *mM*, 25 *mM*, and 30 *mM* for 24 *hr*. Among these three doses of $AlCl_3$, the 20 *mM* concentration decreased cell viability to approximately 50% ($p<0.001$***) compared with untreated cells, and therefore, had a significant cytotoxic effect on rat BM-MSCs (Figure 2A). Furthermore, treatment of MSCs with 50, 100, 250, and 500 *µM* concentrations of crocin could reduce cell death in a dose-dependent manner (Figure 2B). In addition, the cytotoxicity in the pre-treatment group of BM-MSCs with various concentrations of crocin (250, and 500 *µM*) and $AlCl_3$ (20 *mM*) was assessed using the MTT assay. The result showed that crocin (250 *µM*) decreased $AlCl_3$ cytotoxicity by

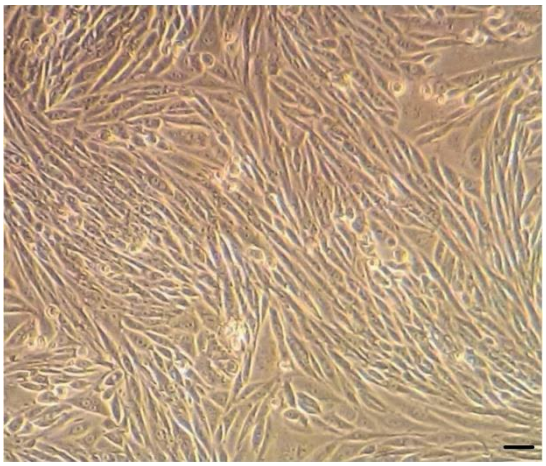

Figure 1. The expansion of rat bone marrow mesenchymal stem cells occurs *in vitro* condition under inverted microscopy (scale bar: 100 *µm*).

Table 1. List of the specific forward (F) and reverse (R) primer (5′→3′) sequences for real-time PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon length (*bp*)
Sox-2	TAAACATGGCAATCAAATGTCCA	TCAAATCCGAATAAATTCCTTCCT	141
E-cadherin	CCTTTGAGGGGTCTCTTGTCC	AAGATGCGATCTCCAGACCC	141
GAPDH	TGCTGAGTATGTCGTGGAGT	CGGAGATGATGACCCTTTTG	95

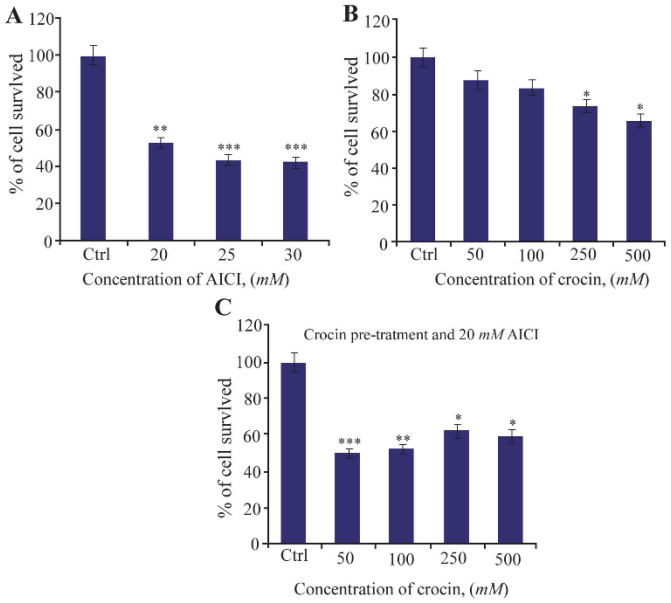

Figure 2. Investigating the effect of various concentrations of crocin, $AlCl_3$ and pre-treatment of crocin and then 20 *mM* of $AlCl_3$ on cell viability and proliferation of BM-MSCs. A) BM-MSCs were treated with different concentration of $AlCl_3$ ranging from 20, 25, and 30 *mM* for 24 *hr*. B) Also the effect of different concentration of crocin (50, 100, 250, 500 μM) on these stem cells for 24 *hr* was defined with MTT assay. C) Moreover, these stem cells were pre-treated with crocin for 24 *hr* then treated with $AlCl_3$ (20 *mM*) showed that crocin 250 μM is proper dose which can decrease the cytotoxicity of $AlCl_3$. Values are Mean±SEM (n=3).
p<0.05*, p<0.01**, p<0.001*** were considered significant between experimental groups with control.

almost 40% compared with the control, which suggests that crocin at dosage of 250 μM exerted a higher survival rate than cells exposed to 500 μM crocin (Figure 2C).

Effect of crocin on mineralization of BM-MSCs exposed to $AlCl_3$

To assay mineralization induced by crocin on rat BM-MSCs exposed to $AlCl_3$, Alizarin red staining was utilized. As shown in figure 3, exposure to $AlCl_3$ (20 *mM*) disrupted the shape of BM-MSCs and the mineralization process. Meanwhile, calcium nodules were increased in a dose-dependent manner in pretreated BM-MSCs with crocin, illustrating an elevation of mineralization compared to the control (untreated cells).

On the other hand, pre-treatment with crocin in a concentration of 250 μM ($p \leq 0.001$***) and 500 μM ($p \leq 0.01$**) resulted in a significantly larger calcium deposit in the treated groups that were pre-treated with crocin and exposed to 20 *mM* of $AlCl_3$. Therefore, pre-treatment with crocin (250, 500 μM) resulted in osteogenesis, which is characterized by significant mineralization following $AlCl_3$ treatment.

Effect of crocin on Sox-2 and E-cadherin mRNA expression in BM-MSCs

Following treatment of BM-MSCs with $AlCl_3$ in concentration 20 *mM* for 24 *hr*, a significant decline in

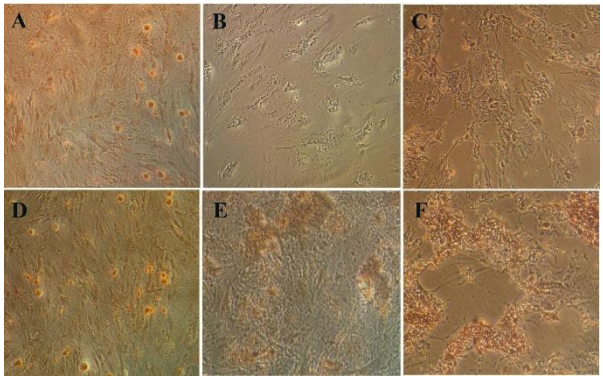

Figure 3. Evaluation of mineralization by Alizarin red staining. The morphology of BMSCs 21 days after treatment period were visualized by inverted microscopy, As shown, suitable concentrations of crocin (250, 500 μM) formed red colored mineral amorphous granules that it was indicating one of the prominent marker differentiations to osteoblasts. A) Control. B) $AlCl_3$ (20 *mM*) exposed BMSCs cells. C-F) BMSCs pretreated with crocin (50, 100, 250, 500 μM) plus exposure to $AlCl_3$ (20 *mM*). Magnification× 200.

Sox-2 and E-cadherin mRNA expression ($p<0.001$***) was observed compared to the control (untreated cells) (Figure 4). However, pre-treatment of BM-MSCs with crocin can mitigate the cytotoxicity of $AlCl_3$, which caused up-regulation in Sox-2 and E-cadherin in BM-MSCs.

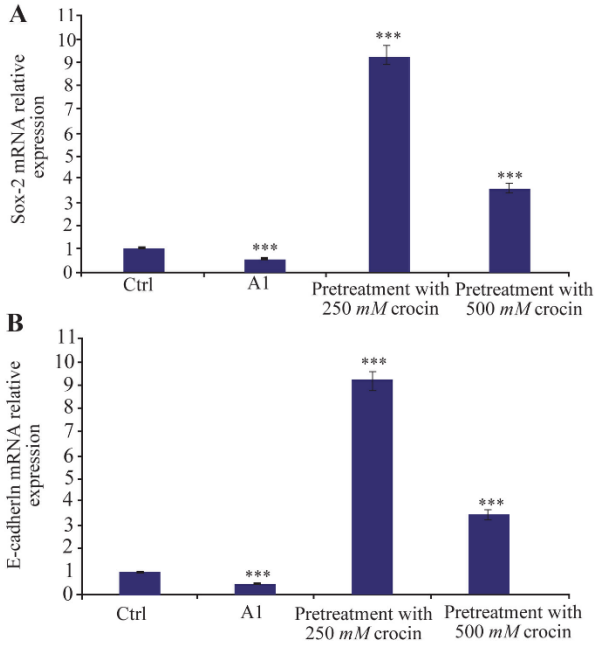

Figure 4. Effect of crocin on mRNA expression of Sox-2 and E-cad induced by $AlCl_3$ treatment of BM-MSCs for 24 *hr*. A) Here, the impact of pre-treatment with 250 and 500 μM concentrations of crocin and 20 *mM* $AlCl_3$ on mRNA expression of Sox-2 in BM-MSCs was defined by real time PCR. B) Also, real-time PCR was performed to determine the E-cadherin mRNA expression level. Values are Mean±SEM (n=3).
p<0.05*, p<0.01**, p<0.001*** were considered significant between experimental groups with control.

Discussion

In this study, we examined the protective effect of crocin against $AlCl_3$-induced cytotoxicity in BM-MSCs. The obtained data showed that $AlCl_3$ reduced cell viability and consequently the mRNA expression of Sox-2 and E-cadherin, which are important for self-renewal and proliferative effects in BM-MSCs, respectively. The results of our current study indicated that the pretreatment of crocin promoted calcium deposit formation, as hallmark of osteogenesis and reduced the cytotoxic effects of $AlCl_3$ on the self-renewal and proliferative effects of BM-MSCs.

MSCs have the ability to self-renew and differentiate into different lineages, and are considered as a possible treatment strategy for various disorders [19]. It is documented that in bone defect disorders, including osteoporosis leads to bone fragility. However, it is also known that MSCs can alleviate bone defect disease by promoting osteoblastic differentiation [20]. Therefore, the identification of effective natural compounds that can accelerate the osteogenic differentiation of BM-MSCs and overcome the toxicity induced by some chemical compounds can be promising in osteoporosis.

Phytochemicals such as polyphenols and flavonoids isolated from green tea and blueberry or catechine or carnosine, have exhibited a proliferative effect on BM-MSCs. In addition, it has been revealed that phytochemicals exert a unique function in regulating the proliferation and differentiation of stem cells *via* signaling pathways such as Runx2, BMP2, and Wnt. Therefore, given the role of these signaling pathways in MSCs proliferation and osteoblast differentiation, herbal compounds have promising therapeutic efficacy in bone defect disorders such as osteoporosis [21].

Wnt signaling has an important role in the self-renewal of stem cells in several tissues. Wnt and β-catenin signaling in osteoporosis has high potential therapeutic value by stimulating self-renewal and osteogenesis [22,23]. MSCs express oct-4, Sox-2, and rex-1 which are stem cell gene markers. Cadherins interact with Wnts, which have a crucial function in MSC biology and other stem cell niches [24]. It is known that Wnt signaling regulates MSC biology by maintaining a balance between self-renewal and terminal differentiation [25]. E-cadherin regulates pluripotency and proliferation of stem cells. It has been shown that changes in β-catenin levels can disrupt the adherens junction and E-cadherin and thus influence the Wnt pathway [13].

Sox transcription factors, which are essential pluripotency markers, have important functions during the development of stem cells and precursor cells, including mesenchymal stem cells [26,27]. Sox proteins can modulate cell type–specific transcription in response to a Wnt signal. In addition, it has been demonstrated that the modulation of Sox proteins is dependent on signaling pathways such as the sonic hedgehog (Shh) and Wnt signaling pathways [26]. Previous studies have reported that Al can induce oxidative stress and also affect metabolism and the expression of various genes by binding to the phosphate group of DNA, RNA, and ATP [10,28]. It has been documented that Al can affect signal transduction pathways such as phospholipase C activity, which can lead to inhibition of IP3, regulation of calcium release and activation of protein kinase. Previous investigations have indicated that inhibition of the Wnt/β-catenin signaling pathway by Al exposure can impair bone formation in rats [29,30]. In addition, Xu *et al*, in 2018 reported findings that confirmed the destruction of osteocytes by Al, mainly *via* oxidative stress-related signaling pathway such as increased levels of c-Jun and p-JNK/JNK ratio as well as recruitment of apoptosis factors [10].

In agreement with these studies, our findings showed the destructive effect of $AlCl_3$ on rat BM-MSCs survival and mineralization. It has been shown that some chemicals such as titanium dioxide and ochratoxin A can suppress the survival, proliferation, and differentiation of stem cells [31,32]. It has been shown that oxidative stress and increased levels of Reactive Oxygen Species (ROS) suppress MSC proliferation and promote senescence. Further, ROS increase can affect MSCs differentiation capacity, such as promoting adipogenesis and diminishing osteogenesis [33].

On the other hand, it has been confirmed that crocin can suppress cell-growth and cell invasion by regulating the Wnt-pathway and E-cadherin [34]. The protective effects of crocin against chronic stress-induced oxidative damage in the rat brain have been previously elucidated. With regard to its antioxidant properties, crocin may be useful against chronic stress-induced oxidative damage by decreasing MDA level as well as increasing GPX, GR, SOD levels and the total antioxidant capacity. Additionally, the effect of crocin against Acrylamide (ACR) induced toxicity on PC12 cells was evaluated and the findings showed that crocin (10-50 μM) up-regulated Bcl-2, Bax as well as attenuation of apoptosis and ROS generation in PC12 cells [35]. Li *et al*, in 2020, demonstrated that crocin promoted bone regeneration in steroid-induced osteonecrosis of the femoral head (SANFH) rat *via* acceleration osteoblast differentiation in BM-MSCs [36]. In accordance, with these results, our finding showed that crocin acts as a protectant against damage induced by $AlCl_3$ on BM-MSCs.

Conclusion

Our results demonstrated that pre-treatment of BM-MSCs with crocin can be suggested to reduce the deleterious effects of $AlCl_3$. Overall, crocin as a plant-derived compound, has the potential to overcome the cytotoxicity induced by $AlCl_3$ and exert a healing function mainly by increasing mineralization and modulating the expression of Sox-2 and E-cadherin, which are important for self-renewal and proliferation potential of BM-MSCs.

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