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

Elaheh Amini 1*, Zahra Baharvand 2, Azadeh Niknejad 3, Yasaman Tabari 4 and Sahel Shemshadi 5

1. Department of Animal Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran
2. Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Department of Cellular & Molecular Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran
4. Department of Cell and Molecular Biology, University of Science and Culture, Royan Institute, ACECR, Tehran, Iran
5. Department of Cell and Developmental Biology, Julius-Maximilians-University, Würzburg, Germany

Abstract

Background: Mesenchymal Stem Cells (MSCs) have the ability to self-renew and proliferate which gives them healing properties in various tissues. Aluminium chloride (AlCl₃) 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 AlCl₃.

Methods: To investigate whether crocin can reduce AlCl₃ 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 AlCl₃ (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 AlCl₃ (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 AlCl₃ 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<0.001).

Conclusion: Overall, the study suggests that crocin can protect BM-MSCs from AlCl₃-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 AlCl₃-induced toxicity.

Keywords: Aluminum chloride, Animals, Cadherins, Crocus, Oxidative stress, Rats

To cite this article: Amini E, Baharvand Z, Niknejad A, Tabari Y, Shemshadi S. The Protective Effect of Crocin on Rat Bone Marrow Mesenchymal Stem Cells Exposed to Aluminum Chloride as an Endocrine Disruptor. Avicenna J Med Biotech 2024;16(2):81-87.

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 osteo-
protective 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.

Some studies have shown that crocin can be effective
in controlling osteoporosis by increasing the num-
ber of T cells (Tregs), increasing the level of anti-
osteoclastogenic cytokines, and reducing the number of
osteoclastogenic Th17 cells. Crocin also uses various
intracellular pathways, including PI3K/AKT and Wnt/β-
catenin signaling pathway. Aluminium (Al) as an
endocrine disruptor is a toxic agent that is widely pre-

cent in water, food additives, therapeutic treatments
and the environment. Endocrine disruptors are sub-
stances that can interfere with various bodily functions,
such as fertility, immune function bone development.
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.

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. MSCs have some important character-
istics such as self-renewal and differentiation, which
make these cells as a valuable source for healing and
inhibiting the progression of diseases such as osteopo-
rosis. These characteristics also make them a promising
candidate for regenerative medicine in osteo-related
diseases.

E-cadherin plays an important role in cell-cell adhes-
sions, which is necessary for the maintenance of cells
and tissues integrity. Recently, it has been demon-
strated that E-cadherin acts as a regulator of pluripo-
tency and self-renewal signaling pathways in stem cells.
Morphological changes resulting from the inter-
ruption of E-cadherin expression can influence cell prolif-
eration and differentiation, which is associated with β-
catenin and Wnt signaling pathway. One of the mod-
ulators 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 pluripo-
tency and plays a distinct role in maintaining the self-
renewal capacity and multipotency of MSCs. Therefore,
in this study, we investigated cytotoxicity of AlCl₃ (Aluminium chloride) and the protective effects of crocin against AlCl₃-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 intraperi-
toneal injection of ketamine (360 mg/kg) and xylazine (30 mg/kg) followed by cervical dislocation. The lum-
ber region to toes were shaved, skin was incised, and
the tibia and femur of both legs were exteriorized as
described earlier. The femur and tibia of both legs
were placed in petri dish containing PBS. The metaph-
ysis region of both bones was cut, then a needle insert-

ed 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 concen-
trate the cells. The supernatant was decanted and 5 ml
of complete media consisting of 85% DMEM (Bio-
idea, Iran), 10% Fetal Bovine Serum (FBS, Bio-idea, Iran), and Penicillin/Streptomycin (100 U/ml) (Bio-
idea, 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 37°C in a humidified atmosphere
of 5% CO₂ 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 sec-
ond and third groups, BM-MSC were treated with var-
ious concentrations of crocin or AlCl₃ for 24 hr. In
fourth group, the cells were pre-treated with various
concentrations of crocin for 24 hr, then treated with
AlCl₃ at IC50 concentration.

Cell cytotoxicity assay

To determine the non-toxic concentration of crocin
(Puyesh Darou Sina, Iran) and the IC50 concentration of
AlCl₃, the MTT assay was performed. In brief, BM-
MSCs were plated in 96-well plates at a density of
5x10³ 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), AlCl₃ (20, 25, and 30 mM) or pre-
treated with crocin (50, 100, 250 and 500 µM) and
then treated with AlCl₃ (20 mM) for 24 hr, in the exper-
mental groups 2 to 4. Each treatment was repeated at
least three times. After 24 hr of treatment, MTT solu-
tion (0.5 mg/ml) (Sigma, USA) was added to the wells
for 3 hr at 37°C. The medium was aspirated, and
the formazan crystals were dissolved in dimethyl sul-
foxide (DMSO, Merck, Germany). Finally, the absorb-
ance was read at 570 nm using iMark™ Microplate
Absorbance Reader (Bio-Rad, USA).

The effect of crocin on mineralization of BM-MSCs ex-
posed to AlCl₃

To evaluate protective effect of crocin on rat BM-
MSCs exposed to AlCl₃, the mineralization of BM-
MSCs was investigated. In this assay, BM-MSCs (pas-
sage 3) were cultured at 2x10³ in 6-well plates. The

Avicenna Journal of Medical Biotechnology, Vol. 16, No. 2, April-June 2024
cells were pretreated with crocin (50, 100, 250, and 500 μM) and then subjected to AlCl₃ (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 AlCl₃ (20 mM) on the transcription level of Sox-2 and E-cadherin of BM-MSCs was evaluated by real-time PCR. First BM-MSCs (2×10⁴) 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 AlCl₃ (20 mM) which has a 50% lethal concentration (IC₅₀) 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 NanoDrop (Thermo Scientific, USA) and cDNA was synthesized 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.

**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 AlCl₃-induced cytotoxicity in BM-MSCs**

In the current investigation, the MTT assay was performed to determination the proper concentration of crocin that can reduce cytotoxic effects of AlCl₃ on BM-MSCs and cause a significant decrease in cell death compared to the control (untreated cells). The IC₅₀ concentration of AlCl₃ was also determined. The obtained data revealed that the cell viability of the BM-MSCs was decreased when cells were exposed to AlCl₃ at concentrations of 20 mM, 25 mM, and 30 mM for 24 hr. Among these three doses of AlCl₃, 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 AlCl₃ (20 mM) was assessed using the MTT assay. The result showed that crocin (250 μM) decreased AlCl₃ cytotoxicity by

![Figure 1. The expansion of rat bone marrow mesenchymal stem cells occurs in vitro condition under inverted microscopy (scale bar: 100 μm).](image-url)
almost 40% compared with the control, which suggests that crocin at dosage of 250 \( \mu M \) exerted a higher survival rate than cells exposed to 500 \( \mu M \) 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 \( \mu M \) 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 \( \mu M \)) 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 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.
Discussion

In this study, we examined the protective effect of crocin against AlCl₃-induced cytotoxicity in BM-MSCs. The obtained data showed that AlCl₃ 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 AlCl₃ 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. 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. 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.

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. 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. It is known that Wnt signaling regulates MSC biology by maintaining a balance between self-renewal and terminal differentiation. 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.

Sox transcription factors, which are essential pluripotency markers, have important functions during the development of stem cells and precursor cells, including mesenchymal stem cells. 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. 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. 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. 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.

In agreement with these studies, our findings showed the destructive effect of AlCl₃ on rat BM-MSC 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. 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.

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. 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. 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. In accordance, with these results, our finding showed that crocin acts as a protectant against damage induced by AlCl₃ on BM-MSCs.

Conclusion

Our results demonstrated that pre-treatment of BM-MSCs with crocin can be suggested to reduce the deleterious effects of AlCl₃. Overall, crocin as a plant-derived compound, has the potential to overcome the cytotoxicity induced by AlCl₃ 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.
Acknowledgement

We thank the staff of the Central Laboratory of Kharazmi University for providing the necessary facilities. This work was supported partially by the research fund for study and research, Kharazmi University (KHU390159172).

References


