

## Investigation of PPAR $\gamma$ Involvement in Crocin Effects on Apoptosis

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**Background:** Apoptosis is an important form of cell death, which is involved in cardiovascular disorders. We investigated the effects of combined crocin and PPAR $\gamma$  antagonist on the expression of regulatory genes involved in apoptosis. Also, we explored whether PPAR $\gamma$  is involved in the protective effect of crocin on apoptosis after myocardial ischemia.

**Methods:** The experimental rats were divided into five groups: control group, Isoprenaline (ISO) group, GW9662 as PPAR $\gamma$  antagonist+ISO, crocin+ISO group, and GW9662+crocin+ISO group. Isoprenaline was injected to induce myocardial ischemia.

**Results:** Findings showed that ISO and GW9662 increased *Bax* and caspase-3 gene expression and cardiac Creatine Phosphokinase (CPK) marker but decreased *Bcl2*. Crocin alleviated the expression of *Bax* and caspase-3 while increased *Bcl2*. It also reduced CPK levels in serum. However, GW9662 reversed these effects.

**Conclusion:** According to results, crocin may regulate apoptosis through PPAR $\gamma$  activation.

**Keywords:** *Bax*, Caspase-3, Creatine Phosphokinase (CPK), Crocin, Myocardial ischemia, GW9662

### Introduction

Necrosis and apoptosis play an essential role in the process of tissue damage which leads to myocardial infarction (Krijnen *et al.*, 2002). Apoptosis is recognized as a process that regulates and requires energy. It is known as a programmed cell death which does not promote inflammatory responses (Saraste & Pulkki 2000). As apoptosis is involved in myocardial infarction and cell damage, understanding this process has therapeutic implications.

Several studies reported that the apoptotic myocytes were abundant in the infarcted zone (Abbate *et al.*, 2000; Olivetti *et al.*, 1996). The regulatory proteins such as *Bcl2* (B-cell lymphoma 2), *Bax* (*Bcl2* associated x, apoptosis regulator), and caspase-3 are involved in apoptosis. *Bcl2* is an inhibitor of apoptosis expressed in the infarcted area, while *Bax* is recognized as a pro-apoptotic protein that triggers caspase cascade events (Ortiz *et al.*, 2003).

Isoproterenol (ISO) is an agonist of the  $\beta$ -adrenergic receptor that results in cell death and heart failure (Zaugg *et al.*, 2000). ISO damages the myocardium by activating apoptotic genes

and oxidative stress. Furthermore, ISO triggers cardiomyocytes' apoptosis through Adenosine Monophosphate Kinase (AMPK) inactivation and endoplasmic reticulum stress (Zhuo *et al.*, 2013).

Antioxidants play some important roles in tissue and organ damages. Crocin is a natural carotenoid compound and water-soluble color pigment and one of the saffron ingredients that has antioxidant (Yaribeygi *et al.*, 2018), anti-apoptotic (Razavi *et al.*, 2016), and antitumor (Sun *et al.*, 2013) effects. Crocin could improve diabetes and metabolic disorders (Abou-Hany *et al.*, 2018; Algandaby, 2020). It exerts protection against lung injury and inflammation (Elsherbiny *et al.*, 2016; Dianat *et al.*, 2018).

GW9662 is known as a Peroxisome Proliferator-Activated Receptor-gamma (PPAR $\gamma$ ) antagonist that is used in many research studies. PPAR $\gamma$  is a subtype of nuclear receptors that has several roles in metabolic disorders and also cardiac damages (Ahmadian *et al.*, 2013; Byeon & Chung 2017). According to studies, PPAR $\gamma$  activation protects against apoptosis-induced oxidative stress by increasing anti-apoptotic gene and protein

expression in cardiomyocytes (Ren et al., 2009). Furthermore, the activation of PPAR $\gamma$  is linked to AMPK and Protein kinase B (AKT) signaling pathways and could inhibit apoptosis (Liu et al., 2016). In this study, we explore the effects of crocin on ISO-induced apoptosis through PPAR $\gamma$ .

## Materials and Methods

Male Wistar rats weighing 200-230 g (3 months old) were used in this study. The animals were housed in an air-conditioned colony room at 23±2°C and under a 12-12 h light-dark cycle and a standard pellet diet and free access to water. The experiments were performed in accordance with the instructions defined by the committee for control and supervision of experiments on animals and approved by the Ethics Committee for Animal Experiments at Jundishapur University of Medical Sciences (IR. AJUMS.ABHC.REC.1397.005).

The rats were divided into five groups: the control group; the ISO group, which received isoprenaline (100 mg/kg) subcutaneously on two terminal days to induce infarction; the G+ISO group, which received GW9662 at a dose of 1 mg/kg intraperitoneally (Mahajan et al. 2017; Agrawal et al. 2014), and then ISO (100 mg/kg) subcutaneously to induce infarction; the C+ISO group, which received crocin (40 mg/kg) (Yaribeyg et al., 2018; Wang et al. 2018) via gavage and then ISO (100 mg/kg) subcutaneously; and the G+C+ISO group, which received crocin (40 mg/kg) and GW9662 (1 mg/kg) and then ISO (100 mg/kg). The rats were treated for 4 weeks.

The serum level of Creatine Phosphokinase (CPK) was measured using an appropriate commercial kit (Okinaka et al., 1961).

## Quantitative Real-Time PCR for Apoptotic Genes Expression

The total RNA from the homogenized tissues was extracted using RNeasy Plus Mini Kit, and then complementary DNA was synthesized. A LightCycler PCR (Roche, Diagnostics) was used to assess the caspase-3, *Bax*, *Bcl2*, and housekeeping genes

levels (Table 1). The gene-specific primers (Bioneer, Daejeon, South Korea) are shown in Table 1. The gene expression level was normalized against the *GAPDH* expression.

## Statistical Analysis

All data were presented as means±SEM. The data analyzed using one-way ANOVA followed by the Tukey post hoc test. P values less than 0.05 were considered statistically significant.

## Results

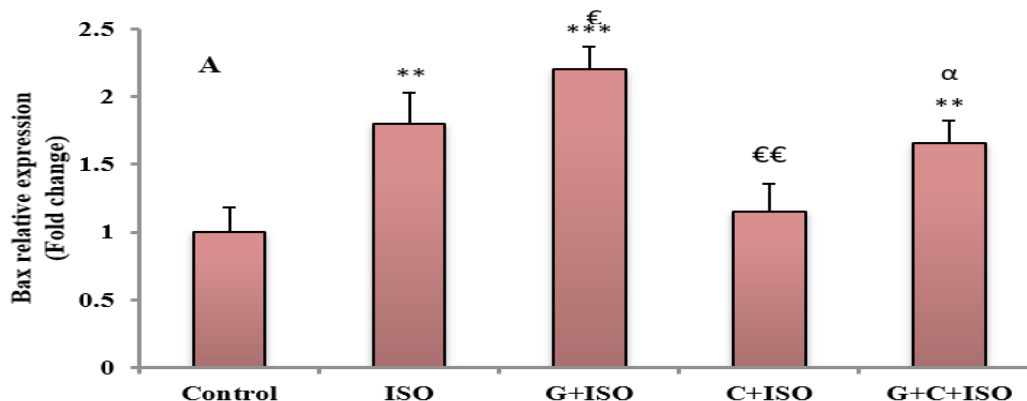
As shown in Figure 1A, the expression of *Bax* was significantly increased in both ISO and G+ISO groups as compared to control (P<0.01, P<0.001, respectively). However, the rising *Bax* expression was more in the G+ISO group. As compared to the ISO group, crocin administration in the C+ISO group significantly reduced *Bax* expression (P<0.01). But, GW9662 significantly reversed the crocin-induced decrease in expression of (P<0.05). As shown in Figure 1B, the caspase expression level was greatly enhanced in the ISO group compared to the control. Although the level of this gene was higher in the G+ISO group than the ISO group, there was no significant difference. Crocin treatment significantly ameliorated caspase-3 compared to the ISO group (P<0.01); however, GW9662 inhibited the crocin effect.

Moreover, *Bcl2* was significantly decreased in both ISO and G+ISO groups as compared to control (P<0.01, P<0.001, respectively) (Figure 1C). Although in the G+ISO group, the reduction was more. Crocin administration significantly augmented this gene expression level compared to the ISO (P<0.01); however, PPAR $\gamma$  antagonist significantly blocked crocin effects.

As shown in Figure 2, CPK was increased in serum of both ISO and G+ISO groups compared to the control. Nevertheless, crocin significantly reduced this marker (P<0.01) compared to the two mentioned groups and reached this factor approximately to the control level, but GW9662 showed the opposite effect of crocin.

Table 1. Sequences of primers

mRNA	Reverse Primer	Forward Primer
<i>Bax</i>	TGAGGTTTATGGCACCTCC	TTTTCTGGGATGAATGGGG
<i>Bcl2</i>	ATCTCCAGTATCCCACTCGTA	TGGTACCTGCAGCTTCTTTC
Caspase-3	ACAAGCCCATTTCAGGGTA	GAGCTTGAACGCGAAGA
<i>GAPDH</i>	CGGAGATGATGACCCTTTTG	TGCTGGTGCTGAGTATGTCGTG



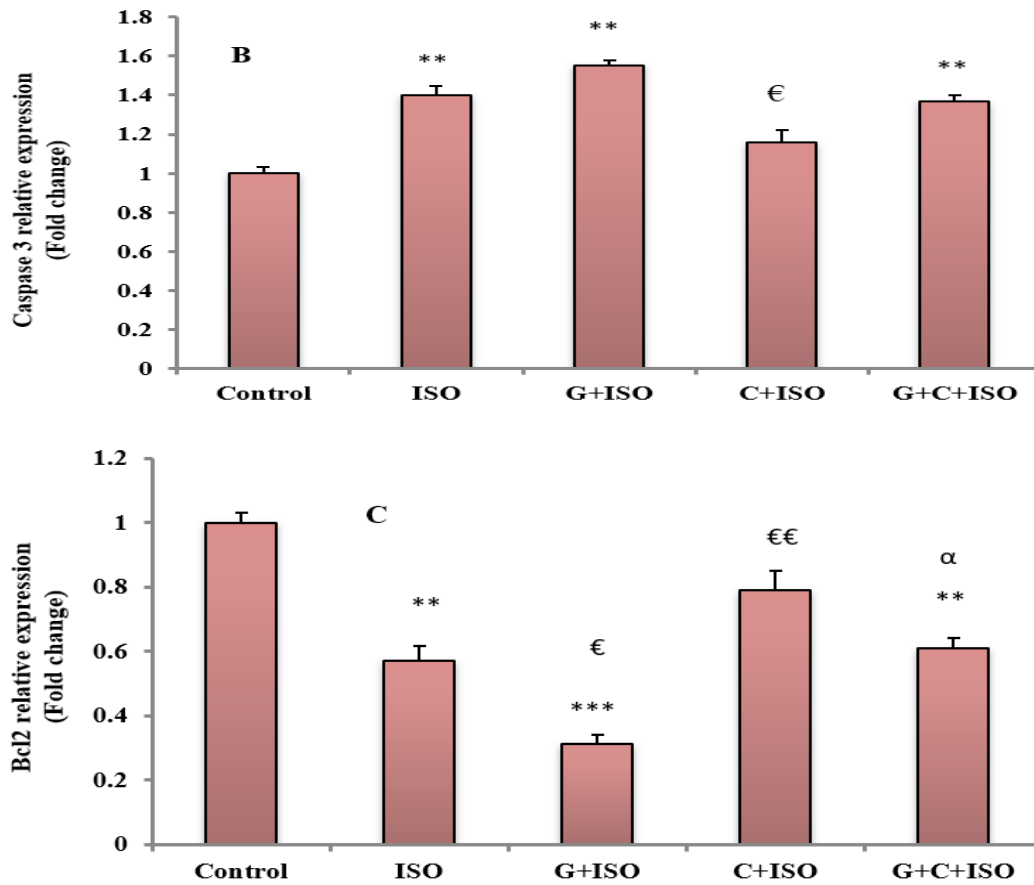


Figure 1: Effect of crocin and GW9662 on the *Bax*, caspase-3, and *Bcl2* genes expression levels.

The data are expressed as the mean±SEM (n=8).

\*\*P<0.01, \*\*\*P<0.001 vs. control; €P<0.05, €€P<0.01 vs. ISO; αP<0.05 vs. C+ISO using one-way ANOVA followed by the Tukey post hoc test.

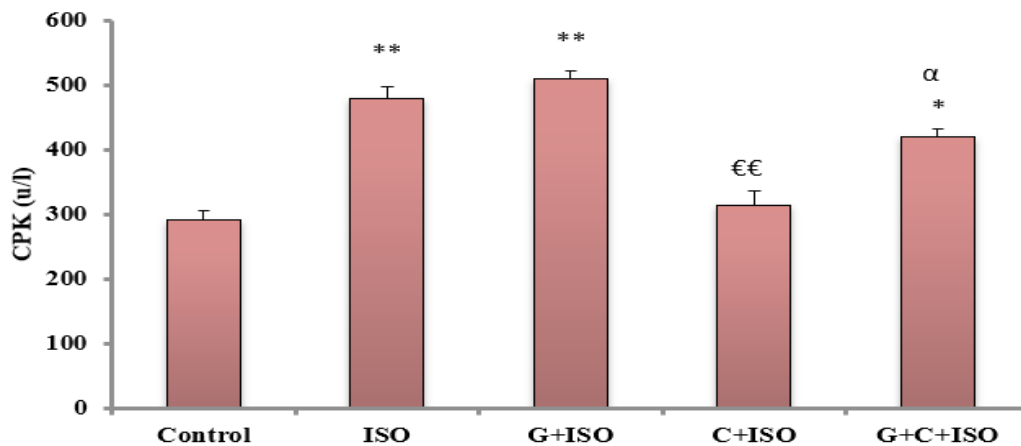


Figure 2: Effect of crocin and GW9662 on CPK content

\*P<0.05, \*\*P<0.01 vs. control; €€P<0.01 vs. ISO; αP<0.05 vs. C+ISO using one-way ANOVA followed by the Tukey post hoc test.

The data are expressed as the mean±SEM (n=8).

## Discussion

This study was performed to determine whether the PPARγ activation is involved in crocin's protective effects in apoptosis after myocardial infarction. The results support that crocin could enhance *Bcl2* and reduced *Bax* and caspase expression levels.

Apoptosis is known as programmed cell death, which involves

a series of cellular regulatory proteins and signals transduction cascade (Fan *et al.*, 2005). Persistent stimulation of β-adrenergic receptors results in mitochondrial dysfunction and oxidative stress that leads to apoptosis. Reactive Oxygen Species (ROS) play an essential role in apoptosis. Cellular redox change is also a part of the signal transduction pathway during apoptosis. Antioxidants can inhibit or delay apoptosis (Amin *et al.*, 2011;

Feng et al., 2020). By preventing oxidant-mediated damage, antioxidants may play a protective role in apoptosis (Zeisel, 2004). They also suppress apoptosis by inhibiting AMPK signaling in cells (Meng et al., 2020).

Because several cellular proteins, including Bax, Bcl2, and caspase, are involved in apoptosis, we measured their expressions. Our study showed that ISO increased expression of Bax and caspase-3 as compared to control, and their expression levels were higher in the G+ISO group than ISO group; crocin treatment significantly reduced Bax and caspase-3 levels. However, in the G+C+ISO group, their expression levels increased compared to the C+ISO group. Bax and caspase 3 are pro-apoptotic proteins. Previous studies reported that Bax expression increased in myocardial infarction (Krijnen et al., 2002). It has been reported that  $\beta$ -adrenergic receptors trigger apoptosis in adult rat ventricular myocytes through ROS/JNK (c-Jun N-terminal Kinases)-dependent activation of the mitochondrial death pathway (Remondino et al., 2003). Similar to our results, a previous study showed that crocin decreased Bax and caspase-3 in cardiotoxicity induced by diazinon (Razavi et al., 2016). It has also been demonstrated that crocin attenuated Bax/Bcl2 ratio and caspase in diabetic cardiomyopathy caused by Streptozotocin (STZ) (Feidantsis et al., 2018). Contrary to our results, crocin stimulated apoptosis by enhancing the Bax and caspase activation in human gastric adenocarcinoma, which could be due to the anti-cancer properties of crocin (Hoshiyar et al., 2013). Furthermore, the current study indicated that the expression of Bcl2 was decreased in both ISO and G+ISO groups, while crocin administration significantly enhanced this gene. *Bcl2* is recognized as an apoptotic inhibitor. According to our findings, a recent research reported that crocin potentiated *Bcl2* expression while ameliorated Bax and caspase expressions in cardiotoxicity induced by arsenic trioxide (Liang et al., 2020). Crocin has also been shown to improve retinal ischemia injury-induced apoptosis via PI3K (Phosphoinositide 3-Kinase)/AKT signaling pathway and it enhanced the Bcl2/Bax ratio (Qi et al., 2013). PPAR $\gamma$  agonists have been shown to improve apoptosis. Rosiglitazone is an agonist of PPAR $\gamma$  that protects cardiac tissue against apoptosis by increasing Bcl2 and reducing caspase in vivo and in vitro studies. It has been demonstrated that cardiomyocytes expressing PPAR $\gamma$  exhibited 3 fold increase in Bcl2 (Ren et al., 2009; Zhang et al., 2020). Also, it has been reported that inactivation of PPAR $\gamma$  causes apoptosis and reduces antioxidants capacity in renal tubular cells (Wen et al. 2016). Also, PPAR $\gamma$  activation has been shown to increase Bcl2 and reduce Bax in a diabetic cardiomyopathy model (Rani et al., 2016). In addition, pioglitazone as PPAR $\gamma$  agonist prevented apoptosis in endothelial cells (Gensch et al., 2007). Therefore, based on several studies, PPAR $\gamma$  has as an anti-apoptotic factor.

Furthermore, CPK was measured to confirm myocardium damage. CPK is a cardiac injury marker that releases in serum during myocardium ischemia. Our results demonstrated that CPK increased in serum of rats in ISO and G+ISO groups while crocin improved this marker's level. Nevertheless, the PPAR $\gamma$  antagonist showed the opposite effect of crocin in the C+G+ISO group. Thus, according to our and other studies, crocin may exert protective effects through PPAR $\gamma$  activation. It has been shown that crocin decreased CPK in cardiotoxicity induced by patulin (Boussabbeh et al., 2015). Our previous study showed that crocin enhanced *PPAR $\gamma$*  gene expression level in diabetic

rats (Badavi et al., 2020).

## Conclusion

Based on the findings, crocin could improve apoptosis by increasing Bcl2 and alleviating Bax and caspase-3. As GW9662 reversed these effects of crocin, therefore, crocin may exert its effects by involving PPAR $\gamma$ .

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## Ethical Permission

This study was approved by the Ethics Committee for Animal Experiments at Jundishapur University of Medical Sciences (IR.AJUMS.ABHC.REC.1397.005).

## Conflict of Interests

The authors declared no conflict of interest.

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