European Review for Medical and Pharmacological Sciences 2021; 25: 4099-4105

The cardioprotective effect of astaxanthin against isoprenaline-induced myocardial injury in rats: involvement of TLR4/NF-κB signaling pathway

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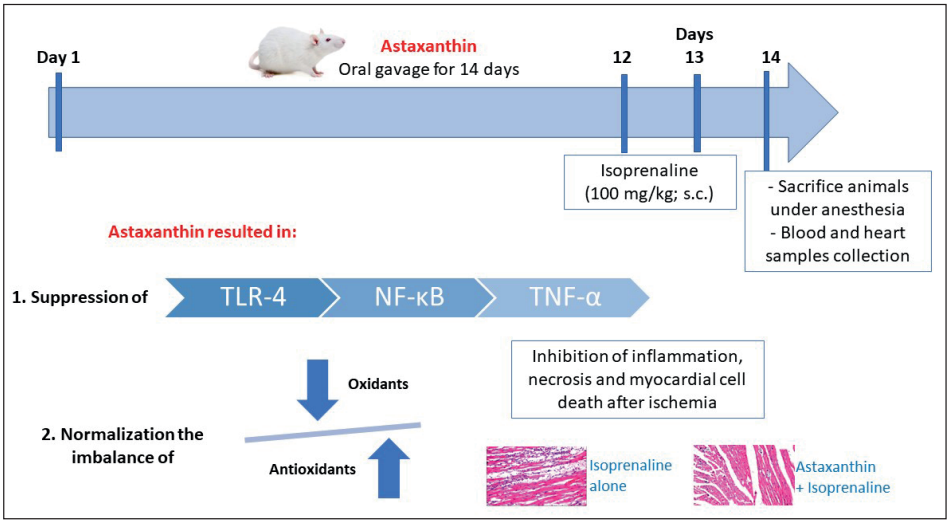
Abstract. – OBJECTIVE: Cardiovascular diseases (CVDs) are a major cause of morbidity and mortality around the world. Nuclear transcription factor kappa B (NF-κB) represents a factor that plays a major role in the pathogenesis of CVDs. The current study aims to investigate the modulatory effects of astaxanthin and its molecular mechanisms in rats with isoprenaline-induced myocardial infarction.

MATERIALS AND METHODS: Rats were pretreated with astaxanthin daily for 14 days prior to inducing myocardial infarction with isoprenaline in the final two days. Blood and heart tissue samples were collected 24 hours after the last dose of isoprenaline was injected for biochemical and histological analysis.

RESULTS: Isoprenaline-induced myocardial injury was demonstrated with histopathological examination of heart tissue and the significantly elevated serum troponin-I. Isoprenaline caused an increase in oxidative stress and a decrease in antioxidants. Toll-like receptor-4 (TLR4), NF-κB and tumor necrosis factor-α (TNF-α) expression levels were significantly higher in infarcted rats. Astaxanthin pretreatment had a significant preventive effect on all of the biochemical and molecular parameters tested in myocardial infarcted rats.

CONCLUSIONS: Astaxanthin's cardioprotective effect has been linked to the inhibition of the TLR4/NF-κB signaling pathway. This inhibits the release of inflammatory cytokines, which can cause myocardial cell death. Because of its antioxidant and anti-inflammatory properties, astaxanthin is a promising cardioprotective agent.

Key Words:
Astaxanthin, Cardioprotective, Toll like receptor-4, Nuclear factor-κβ, Tumor necrosis factor-α.



Graphical Abstract.

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Introduction

Cardiovascular diseases (CVDs) are a major cause of morbidity and mortality worldwide. Acute coronary syndromes are a leading cause of sudden cardiac death. According to the WHO, CVDs were responsible for approximately 20 million deaths in 2015[1]. A sequence of pathologies that are induced and augmented by molecular mechanisms are involved in the complex physiopathological events of acute myocardial infarction. Nuclear factor-B activation for an extended period has been shown to play a critical role in the occurrence of myocardial cell death. It causes chronic inflammation by releasing cytokines such as interleukin-6, interleukin-1, and tumor necrosis factor, which causes endoplasmic reticulum stress responses and, eventually, cell death[2,3]. Recently, the toll-like receptors (TLRs) relation with the induction of myocardial inflammation and death was studied. TLRs were reported to be highly expressed in the myocardial infarcted cells. It was supposed to be an important activator of many transcription factors like nuclear factor-κB[4]. As a result, the regulation of these molecular mechanisms has been thoroughly investigated in the hope of identifying them as a potential target that can promote myocardial cell survival while suppressing cell death.

Astaxanthin is a keto-carotenoid red pigment from the carotenoids subclass; xanthophyll[5]. Astaxanthin has a high antioxidant capacity and a cell signal modulating effect, making it useful in combating oxidative stress-related diseases. These include neuronal damage prevention, cardioprotective effects, anti-aging and anticancer activity, and skin damage prevention with UV radiations[6,7]. Based on astaxanthin's unique chemical features and its supposed ability to suppress the TLR4 signaling pathway and inhibit the subsequent inflammatory responses, it is anticipated as a suitable preventive and therapeutic agent in cardiovascular diseases. Hence, the current study sought to investigate the cardioprotective and therapeutic potential of astaxanthin against experimentally induced myocardial infarction in rats, as well as the molecular mechanisms underlying these effects.

Materials and Methods

Animals

The current study included male Wistar albino rats weighing between 150 and 200 g. These rats were obtained from the Egyptian Company to produce Vaccines, Sera, and Drugs (EGYVAC; Cairo, Egypt). Rats were housed in plastic cages at the animal house of October University for Modern Sciences and Arts under constant conditions (temperature 25±3ºC and humidity 50%). There was free access to water and standard pellet chow.

Drugs and Chemicals

Astaxanthin was obtained from Jarrow Formulas (Los Angeles, CA, USA); whereas isoprenaline hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals used were of the analytical grade.

Induction of Myocardial Infarction

For the induction of myocardial infarction, isoprenaline HCl (100 mg/kg) dissolved in saline was injected subcutaneously for two days in a row. The route and dose were chosen based on previous research[8].

Experimental Design

Rats were categorized into three groups (n=6) at random. The first set of rats served as the standard control group. Isoprenaline HCl (100 mg/kg; s.c.) was given to an isoprenaline control group to induce myocardial infarction. The third group was given astaxanthin dissolved in corn oil (10 mg/kg; p.o.) for 14 days before being given isoprenaline injections the last two days. Based on previous research, the dose and route of administration of astaxanthin were determined[9].

Blood samples were collected via the retro-orbital plexus at the end of the experiment for analysis of serum troponin-I. The rats were then sacrificed via cervical dislocation under urethane anesthesia, and their hearts were quickly dissected out and washed in ice-cold saline. The isolated hearts were used for biochemical assessment of lipid peroxides expressed as malondialdehyde (MDA) and superoxide dismutase activity (SOD), in addition to qRT-PCR expression of tumor necrosis factor-alpha (TNF-α), nuclear factor-kβ (NF-kβ) and toll like receptor-4 (TLR-4). Sections of the isolated hearts fixed in formalin were used for the histopathological examination of the myocardium.

Biochemical Investigations

The activity of CK-MB in serum was determined using Stanbio CK-MB diagnostic kit (Boerne, TX, USA). Lipid peroxidation in heart tissues was assessed by measuring thiobarbituric acid reactive substances content evaluated as malondialdehyde (MDA) in heart homogenate using

Table I. Sequences of the used primers.

Gene	Forward primer	Reverse primer
TLR-4	5′-ATATTGACAGGAAACCCCATCCA-3′	5′-AGAGAGATTGAGTAGGGGCATTT-3′
NF-κβ	5′-ATGGCAGACGATGATCCCTAC-3′	5′-CGGAATCGAAATCCCCTCTGTT-3′
TNF-α	5′-CCCACTCTGACCCCTTTACT-3′	5′-TTTGAGTCCTTGATGGTGGT-3′
GAPDH	5′-CTGGAGAAACCTGCCAAGTA-3′	5′-TGTTGCTGTAGCCGTATTCA-3′

Biodiagnostic (Cairo, Egypt) standard kit. SOD activity was determined using a Biodiagnostic (Cairo, Egypt) standard kit.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

The hearts were used for total RNA isolation using TRIzol (Invitrogen; Auckland, *New Zealand*) along with the instructions of the manufacturer. Using the Reverse Transcriptase M-MLV (Promega; Madison, WI, USA), RNA was reverse-transcribed into cDNA. Sequences of the primers that were used are shown in Table I.

Quantitative reverse transcriptase PCR was completed by using a Power SYBR Green PCR Master Mix on the CFX96 Instrument (Bio-Rad; Hercules, CA, USA). The final extension at 72°C incubation was continued for a further 10 min GAPDH was used as housekeeping gene. The relative quantification of target gene was done by the $2^{\Delta\Delta CT}$ formula method[10].

Histopathologic Assessment of Myocardial Tissue Damage

The hearts of rats from the different groups were fixed in 10% formalin solution. Heart tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin & eosin (H&E) stain for routine examination for histopathological examination using the electric light microscope. This is according to the method previously described by JD Bancroft and M Gamble[11].

Statistical Analysis

Data are presented in the form of mean ± SEM. The comparisons among means of different groups were done via one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons posttest[12]. Kruskel-Wallis test was used to analyze the histopathological scores and followed by Dunn's multiple comparisons test. The level of significance was taken as $p < 0.05$. All the statistical tests carried out using GraphPad Prism software package, version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Isoprenaline injection successfully produced significant myocardial injury, as evidenced by a significant increase in serum troponin-I levels when compared to normal rats (Table II). In addition, in the isoprenaline control rats, histological examination revealed multiple focal areas of degenerated myocardial cells with inflammatory cell infiltration in a diffuse manner all over the myocardial bundles (Figure 1). Pretreatment with astaxanthin provided significant protection against the deleterious effects of isoprenaline on the myocardium, as demonstrated by a significant decrease in serum troponin-I levels in the astaxanthin-treated group (Table II). The histological examination showed only a few focal narrow areas of degeneration with few inflammatory cells' infiltration in the astaxanthin group when compared to the isoprenaline control rats (Figure 1). Isoprenaline induced considerable oxidative stress, as evidenced by a significant increase in MDA content in the heart and a significant suppression of SOD activity. Astaxanthin was a powerful antioxidant that successfully reduced cardiac lipid peroxides while increasing antioxidant SOD activity in heart tissue (Table II).

The increased expression of the inflammatory and pro-inflammatory cytokines upon isoprenaline injection was detected through the significant increase in the expression of TLR-4, NF-κβ, and TNF-α when compared to the normal group. On the other hand, pretreatment with astaxanthin produced significant suppression of TLR-4, NF-κβ, and TNF-α expression in heart tissue as compared to the isoprenaline control rats (Figure 2, Table II).

Discussion

Isoprenaline HCl as a powerful β-adrenergic agonist increases the myocardial oxygen demand via its positive inotropic and chronotropic actions.

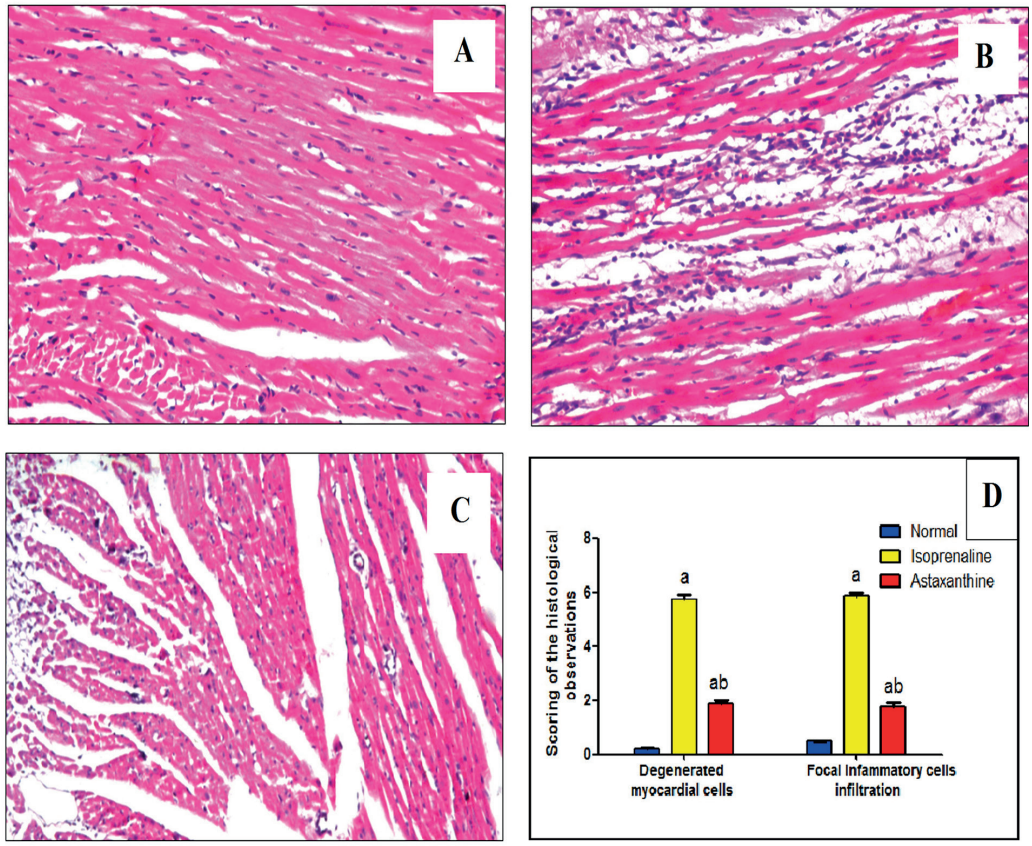

Figure 1. Effect of astaxanthin treatment on the histological structure of the heart tissue in rats with isoprenaline-induced myocardial infarction (H&E×16). **A,** Showed the normal histological structure of the myocardial bundles with one centrally nucleated cardiomyocytes in normal rats. Isoprenaline injection produced multiple focal areas of degenerated myocardium with inflammatory cells infiltration in a diffused manner all over the myocardial bundles (**B**). On the other hand, pretreatment with astaxanthin produced a few focal narrow areas of degeneration with a few inflammatory cells' infiltration (**C**). Scoring of the histological observations in the myocardium is presented in (**D**).

Table II. Effect of astaxanthin treatment on serum troponin-I level and cardiac contents of tumor necrosis factor-alpha (TNF-α), malondialdehyde (MDA) and superoxide dismutase (SOD).

Parameter Groups	Troponin-I (pg/ml)	TNF-α (pg/g wet tissue)	MDA (µmol/g wet tissue)	SOD (pg/g wet tissue)
Normal control	78.6 ± 4.83	99.06 ± 11.16	0.32 ± 0.02	280.20 ± 23.73
Isoprenaline control	505.0^{a} ± 33.66	1179^{a} ± 118.1	1.82^{a} ± 0.22	40.18^{a} ± 5.77
Astaxanthine + Isoprenaline	159.2^{ab} ± 2.44	285.7^{b} ± 31.31	0.37^{ab} ± 0.02	624.80^{ab} ± 58.14

Each value represents mean ± SEM (n = 6). a, Significantly different from normal control group at $p < 0.05$. b, Significantly different from isoprenaline control group at $p < 0.05$.

Using high doses of isoprenaline in experimental animal models results in hearts with infarcted-like lesions resembling those present in patients with myocardial infarction[13]. Myocardial infarction was experimentally-induced in the present study using subcutaneous injection of high doses of isoprenaline HCl (100 mg/kg/day) for two constitutive days in rats. Diverse mechanisms explaining isoprenaline-induced cardiac damage include generation of highly cytotoxic free radicals, increased calcium overload, mitochondrial injury, or dysfunction in addition to the proin-

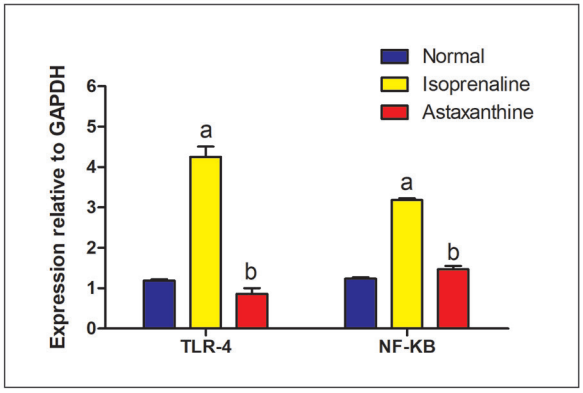

Figure 2. Effect of astaxanthin treatment on the expression of toll like receptor-4 (TLR-4) and nuclear factor-κβ (NF-κβ) in heart tissue. Each value represents mean ± SEM (n = 6). **A,** Significantly different from normal control group at $p < 0.05$. **B**, Significantly different from isoprenaline control group at $p < 0.05$

flammatory status, and alteration of the balance between pro-inflammatory and anti-inflammatory factors[13,14].

There is a strong association between oxidative stress and myocardial infarction injury. The imbalance in the metabolism of oxidants/antioxidants was reported in acute myocardial infarction patients. Enzymes that can scavenge free radicals, such as SOD, CAT, GSH as well as GPX, are considered as the first line cellular defense for oxidative damage[15]. A significant increase in MDA and a decrease in the antioxidant SOD in heart tissue of isoprenaline control rats was observed in the current study. These results are in line with the previous studies that suggest the involvement of oxidative stress in isoprenaline-induced myocardial infarction in rats. While treatment with astaxanthin successfully increased the antioxidant activity and decreased lipid peroxidation. Several previous studies highlighted the possible potent antioxidant activities of astaxanthin through its conjugated double bonds at its center giving it the antioxidant effects[16]. The antioxidant properties of astaxanthin are related to its chemical and physical interactions, as it contains a polyene chain and multiple double bonds. This structure has the ability to reduce singlet oxygen and radicals within cells. Astaxanthin's polyene chain scavenges free radicals in the cell membrane[17].

The pro-inflammatory component stands in the first line from the pathological involved factors in the development and augmentation of cardiovascular diseases. While inflammation can be beneficial for defence and tissue-remodeling mechanisms, it is also responsible for the occurrence of myocardial cell damage when it becomes overexpressed or chronic[2].

Toll-like receptors (TLRs) are a type of pattern recognition receptor that has been linked to cardiovascular disease. Several studies have revealed the ability of TLR4 to activate several pro-inflammatory cytokine genes expression which can further play an essential role in myocardial inflammation, mainly myocarditis, myocardial infarction, and ischemia-reperfusion injury as well as heart failure. TLR4 is reported to be an emerging important target for anti-inflammatory treatments[18]. The current study demonstrated the vital role of TLR4 in the pathogenesis of myocardial cell injury being highly expressed in the hearts of the isoprenaline-infarcted rats. Targeting TLR4 is one of the main goals of astaxanthin treatment, which significantly reduced its expression in the astaxanthin-treated group's hearts compared to the isoprenaline infarcted control group. In a model of subarachnoid hemorrhage, astaxanthin was shown to suppress the TLR4 signaling pathway and inhibit subsequent inflammatory responses[19], hence it was suggested to offer a promising cardioprotective effect.

The activation of TLR4 induces signaling pathways that activate several transcription factors, like nuclear factor-kappa B (NF-κB), and subsequently induce the production of pro-inflammatory cytokines[18]. NF-κB represents an important factor involved in the molecular mechanisms that lay on the basis of cardiovascular diseases[20]. Regarding its mechanism of action, NF-κB is responsible for the regulation of pro-inflammatory cytokines and their modulation inside the cardiac tissue, especially during I/R injury. In this context, chronic activation of NF-κB can result in further damaging consequences, including cardiac cell death[2].

The current study revealed highly increased expression of NF-κB in the hearts of the control rats and normal NF-κB expression upon astaxanthin treatment. Astaxanthin is reported to have anti-inflammatory effects through suppressing the NF-κB pathway[21].

Furthermore, the present study demonstrated that once the TLR4/NF-κB signaling is activated, TNF-α production is highly increase triggering further inflammatory events. TNF-α has been shown to play a key regulatory role in the inflammatory response. It contributes to the incidence of myocardial infarction injury by stimulating necrosis, apoptosis, and autophagy[22]. Furthermo-

re, TNF-α was reported to stimulate the NF-κB expression, resulting in a vicious cycle of inflammatory reactions[23]. Astaxanthin-treated rats showed a significant decrease in the neutrophils' infiltration in the myocardium with subsequent attenuation of the inflammatory response. This effect was revealed by a significant decrease in TNF-α expression in the hearts of the treated rats. Previous studies have attributed the medical applications of astaxanthin to its role in the inhibition of cytokines production and inflammatory genes expression[24].

When compared to the isoprenaline control group, the cardioprotective effect of astaxanthin was reflected in the current study by the marked suppression of the elevated troponin-I level and the almost normal myocardium histological structure after pretreatment with astaxanthin.

Conclusions

Astaxanthin appears to be a promising cardioprotective agent against various cardiovascular diseases including myocardial infarction. The current study demonstrated that astaxanthin can suppress the inflammatory-induced myocardial injury by suppressing the TLR4/NF-κB signaling pathway with further inhibition of the release of the master pro-inflammatory cytokine TNF-α. Also, it can successfully provide further tissue protection via preserving the oxidant/antioxidant balance that is related to its unique structure. These conclusions are illustrated in the **Graphical Abstract**.

Compliance with Ethical Standards

The study was done according to the ethics standards and approved under the code RSPHO2.1 (2019) from the ethics committee of the October University for Modern Sciences and Arts, Egypt.

Conflict of Interest

The authors have declared that they have no potential conflicts of interest in relation to the research, authorship, or publication of this article.

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Acknowledgments

The authors would like to thank Dr. Adel M. Bakeer, Professor of Pathology at Cairo University's Faculty of Veterinary Medicine, for his assistance in examining and interpreting the histopathologic aspects of the current study.

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