The Antioxidant Potential of Astaxanthin on Arsenic Trioxide Induced Cardiac Damage in Male Wistar Rats

Binu P¹, Mathews V Varghese¹, Manju Alex¹, Abhilash S¹, Vineetha R C¹, Harikumaran Nair R¹*

¹(School of Biosciences, Mahatma Gandhi University, Priyadarshini Hills P.O., Kottayam, Kerala, India - 686 560)

Abstract: Arsenic is an environmental toxic metal implicated in human diseases. Carotenoids are the most widespread group of pigments found in nature. Astaxanthin is a xanthophylls keto carotenoid with antioxidant property and commonly found in marine environment. The purpose of this study was to examine a possible cardioprotective effect of astaxanthin against arsenic induced toxicity in a rat model. Twenty four male wistar rats were randomly divided into four experimental groups, as follows: control group, arsenic trioxide treated group (4 mg/kg body weight), astaxanthin treated group (50 mg/kg body weight) and arsenic was co-administered with astaxanthin (4 mg/kg body weight + 50 mg/kg body weight) for a period of 30 days. In experimental rats, oral administration of arsenic trioxide significantly induced cardiac damage which was evident from the increased levels of serum lactate dehydrogenase (LDH), creatine kinase (CK), calcium level with a significant (p< 0.05) decrease in level of potassium. Arsenic treatment also significantly decreased the activity of non enzymatic antioxidant reduced glutathione (GSH) level in myocardium. A markedly increased level of malondialdehyde (MDA), an index of lipid peroxidation was shown in arsenic treated group. Co-administration of astaxanthin along with arsenic trioxide resulted in a reversal of arsenic induced biochemical changes in heart accompanied by a significant decrease in lipid peroxidation and an increase in the level of antioxidant defense system. The histopathological study in the heart tissue of experimental rats also shows that astaxanthin markedly reduced the toxicity of arsenic and preserved the normal histological architecture of the heart tissue. In conclusion, astaxanthin treatment has a protective effect against arsenic trioxide induced cardiac damage and oxidative stress in a rat model.

Keywords: Astaxanthin, Arsenic trioxide, Oxidative stress, Myocardium, Antioxidant

I. Introduction

Carotenoids are ubiquitous in nature and present in plants, algae and microorganisms. There are two class types of carotenoids based on their chemical composition: carotenes and xanthophylls [1]. Astaxanthin is a carotenoid it belongs to a larger class of phytochemicals known as “terpenes” which are built from 5 carbon precursors; isopentenyldiphosphate (IPP) and dimethylallyldiphosphate (DMAP). Astaxanthin is a xanthophylls keto carotenoid which contains two additional oxygenated groups on each ring structure compared with other carotenoids, resulting in enhanced antioxidant properties [2]. The US FDA approved astaxanthin in 1987 as a feed additive for the aqua industry and in 1999 approved it as a dietary supplement. All of which suggest a potential role for this carotenoid in the prevention of cardiovascular diseases [2]. Astaxanthin is a xanthophylls keto carotenoid and commonly found in marine environment. This compound occurs naturally in a wide variety of living organisms including microalgae (Haematococcuspluvialis, Chlorella zofingiensis, and Chlorococcumsp.), fungi (red yeast Phaffiarhodozyma), seafood, and some birds such as flamingos and quail; it is reddish-coloured, and gives salmon, shrimp and lobster their distinctive colouration. The microalga H. pluvialis has the highest capacity to accumulate astaxanthin up to 4–5% of cell dry weight [3].

We used arsenic trioxide (AS₂O₃) as an inducing agent for cardiac toxicity. Arsenic (AS), widely distributed semi-metallic element occurring in various compounds in the crust of the earth is considered one of the most significant hazards in the environment. Arsenic has been used since ancient times as a therapeutic agent. It is a promising drug for patients with relapsed acute promyelocytic Leukemia (APL) [4]. But its clinical efficacy is burdened by the serious cardiac toxicities. The toxic effect of arsenic trioxide in heart and liver tissue was already studied in our lab. Toxic effects of inorganic arsenic include denaturing of cellular enzymes through interaction with sulphhydryl groups [5] causing cellular damage through increased reactive oxygen species (ROS) [6]. Arsenic trioxide induced cardiac toxicity is mainly included the alterations of DNA repair and methylation, generation of reactive oxygen species, change in cardiac ion channels and apoptosis [7]. This study was designed to investigate protective efficacy of xanthophyll carotenoid astaxanthin on As₂O₃ for a period of 30 days continuous oral administration in male wistar rats.
II. Materials And Methods

Chemicals
Astaxanthin (Ambe, phytoextract Pvt. Ltd, Gujarat), Arsenic trioxide, Sodium pyruvate, GSH, phenaninemethosulphate (PMS), sodium citrate, nitroblue tetrazolium (NBT) were obtained from Sigma – Aldrich, Bangalore, India. L – aspartate, α –oxoglutarate, 2,4- dinitrobenzine and benzoic acid (DTMB ), NADH, 1- anilinonaphthalene-8-sulfonic acid ammonium salt (ANSA), thiobarbituric acid (TBA), NADPH, 1-chloro2,4 dinitro benzene (CDNB) were purchased from Merk Specialties Pvt, Ltd, Mumbai, India. Ketamine hydrochloride and Xylazine hydrochloride (Sigma Aldrich, Pvt, Ltd, Mumbai, India).

Animal treatment
Twenty four male Wistar rats weighing 180-250 g were purchased from the small Animal Breeding Station (SABS) of Govt. Veterinary College, Mannuthy, Thrissur, and Kerala, India and acclimatized for six days. All the animals were maintained under standard laboratory conditions of temperature (25± 2˚C) and 12 hour light and dark cycles throughout the experiment period. The rats were provided with laboratory chow (Hindustan Lever Ltd. India) and tap water ad libitum. Experiments were conducted as per the guidelines of Institutional Animal Ethical committee, School of Biosciences, M G University, Kottayam

Experimental design
The rats were divided in to 4 groups containing six rats in each group and were treated as follows:
Group I - Normal control.
Group II - Oral administration of single dose of 4mg /kg body weight of arsenic trioxide every day for a period of 30 days.
Group III - Oral administration of single dose of 50 mg/kg body weight of astaxanthin every day for a period of 30 days.
Group IV - Oral administration of single dose of astaxanthin and arsenic trioxide every day for a period of 30 days.
After that, experimental animals and control rats were decapitated, blood was collected and centrifuged at 3000 rpm for 20 minutes. The clear supernatant was used for the estimation of serum enzymes and other biochemical parameters. The heart tissue was carefully directed for the determination of lipid per oxidation, antioxidant status and histopathology.

Biochemical analysis
The serum Lactate dehydrogenase (LDH), Creatine kinase (CK) calcium and potassium activities were estimated by using commercially available kits (AGAPPE Diagnostics Ltd., Kerala, India).

Preparation of tissue homogenate
The dissected heart sample was rinsed with ice cold isotonic saline and dried using after filter paper. 10% homogenate was prepared in Tris- HCL buffer (0.02M, pH= 7.4) and centrifuged at 1000 rpm for 20 minutes at 4˚C. The clear supernatant was used for the assay of lipid per oxidation and antioxidant system.

Assay of tissue GSH
GSH was measured in tissue homogenate according to the method described by Ellman [8]. In the assay mixture contained 0.1 mL of sample, 0.85 mL of PBS (0.3 M, pH 7.4), and 0.05 mL of DTNB (10 mM). The reaction was read at 412 nm, and results were expressed as μmoles of GSH/g protein.

Assay of tissue TBARS
Thiobarbituric acid reactive substances (TBARS) activity was estimated by the method of Beuge and Aust [9]. That was measured after the addition of 2 mL of TBARS (15% w/v trichloroacetic acid and 0.25 N of HCl) to 1 mL of heart tissue homogenate, then heated in a boiling water bath for 15 minutes. After cooling, the suspension was centrifuged at 1000 rpm for 10 minutes. The supernatant was then separated, and absorbance was measured at 535 nm. The results were expressed as nM of TBARS formed/mg protein.

Histopathology
Heart tissue samples were fixed in 10% formalin. After fixation tissues were processed and embedded in paraffin. 5 micro thick sections stained with hematoxylin and eosin was examined under Phase contrast microscope (Inea, Olympus).
Statistical Analysis

Data were obtained from repeated experiments and the results were represented as mean ± standard deviation (SD). The results obtained from the experiments were analyzed using the statistical program Origin, version 7 (Origin Lab Corporation, Northampton, USA).

III. Results And Discussion
Arsenic induced changes of cardiac function markers and the protective role of astaxanthin are shown in Fig. 1 & 2. Oral administration of arsenic resulted in significantly increased activity of LDH and CK (p<0.05) compared to controls, whereas co-administration of eugenol exhibited a significant decrease in these cardiac marker enzymes (p<0.05) compared to the As₂O₃ treated group.

The variation in the normal level serum electrolytes calcium and potassium in different experiment groups were shown in Fig.3. In arsenic treated rats showed significantly (p<0.05) high calcium and low potassium level compared with that of normal control rats. Co-administration of astaxanthin along with arsenic significantly (p<0.05) restored the levels of serum calcium and potassium when compared with arsenic treated rats. The oral treatment with arsenic (4 mg/kg b.wt) for a period of 30 days significantly (p<0.05) decrease in the activities of tissue reduced glutathione (GSH) level compared with normal control (fig.4) Furthermore astaxanthin antioxidant molecule significantly enhanced the non enzymatic antioxidant GSH level.

The Fig (5) depicts the level of cardiac lipid peroxidation marker MDA level. The level of MDA in arsenic administered rats were significantly (p<0.05) higher than control rats and decreased significantly (p<0.05) in the arsenic trioxide + astaxanthin group compared with the arsenic trioxide group.
Histology of Heart

Haemotoxylin and eosin staining of the heart tissue showed significant structural abnormalities such as oedema, fibre separation, early necrotic changes and focal hemorrhage in arsenic trioxide treated rats (Figure:B). Astaxanthin co-treated rats (Figure:D) only mild cardiac changes were observed. Heart tissue of normal control (Figure: A) and Astaxanthin control (Figure: C) showed cardiac tissue with normal morphology such as inter branching myocardial fibers, coronaries and nucleus.

The main objective of this study was to investigate the hypothesis that astaxanthin has a protective effect against As$_2$O$_3$ myocardial injury. In the present study, we describe that oral administration of arsenic induces cardiac dysfunction, assessed by the increased level of cardiac diagnostic marker enzymes such as LDH and CK, high calcium and low potassium concentration in serum, decreased level of tissue GSH and increased concentration of MDA. These data agree with previous studies on both arsenic and astaxanthin from our laboratory[10,11]. The results obtained in this study revealed that astaxanthin is a potent inhibitor of arsenic induced heart tissue injury.

Lactate dehydrogenase and creatine kinase are two major cardiac markers because of its tissue specificity and catalytic activity. Lactate dehydrogenase (LDH) is an isoenzyme catalyses the interconversion of lactate and pyruvate. In arsenic treated group the level of these two enzymes were increased compared with the normal control rats. Arsenic trioxide increases LDH leakage from cytoplasm and consequently causes necrosis in cardiomyocytes. In most toxicological studies, LDH release is a convenient screening method for assessment of irreversible cellular injury. Arsenic directly inhibits the LDH activity may be due to damage of cell and thus led leakage of this enzyme in to serum [12]. Manna et al [13] reported that plasma level of LDH activity was increased during the exposure of arsenic. The enzyme creatine kinase (CK) catalyses the conversion of creatine and consumes ATP to create phosphocreatine and adenosine di phosphate (ADP). In cardiac muscle, the significance of the different isoforms of CK has been related to their intracellular localization rather than to kinetic differences [14]. Shaik et al [15]observed that these enzymes are leaked out low oxygen supply to heart causing myocardial infarction. In astaxanthin cotreated group there is significance change in parameters like LDH and creatine kinase as compared with arsenic treated group. This may be due to the cardioprotective nature of astaxanthin by scavenging the free radicals during arsenic treatment.

Arsenic administration results significant elevation of extracellular calcium and decreased potassium level. Calcium is an important second messenger and its disruption can activate pathways that lead to apoptosis.
[16]. Arsenic induced apoptosis in leukemia cell is associated with excessive Ca$^{2+}$ influx and overload [17]. Pinton et al [18] reported that free radical over production increase calcium concentration Ca$^{2+}$ overload results in mitochondrial calcium accumulation and induction of apoptosis. Calcium ions are secondary in numerous signaling pathways especially in cardiac myocytes. Arsenic caused the Ca$^{2+}$ - dependent production of superoxide. There are different mechanisms by which ATO could induce calcium overload through the generation of ROS. It has been reported that a burst of ROS increases the Ca$^{2+}$ influx through heart specific L–type calcium channel (LTCC) and leads to cytosolic Ca$^{2+}$ accumulation [19]. The increased rate of intracellular calcium content would trigger calcium release from the sarcoplasmic reticulum, which results in an increase in contraction of cardiac muscle. In cardiac tissue, elevation of calcium has been linked to various abnormalities such as ventricular arrhythmias and contractile dysfunction. During arsenic treatment potassium concentration was significantly declined this may be due to alteration in electrolyte balance. Hypokalemia can occasionally provoke cardiac arrhythmias. But cotreatment with the carotenoid astaxanthin normalized the level of both calcium and potassium. This is a positive indication for astaxanthin as a cardioprotective through ionic concentration maintenance.

The result showed during arsenic administration reduced the amount of reduced glutathione (GSH) which was compared with the normal control rats. GSH is very important in maintaining the cellular redox status. Depletion of this main non protein antioxidant in the cell GSH is considered a marker of oxidative stress [20]. It was able to clear away the superoxide anion free radical and provide electrons for enzymes that reduce H$_2$O$_2$ to H$_2$O. It has been reported that the intracellular GSH content has a decisive effect on arsenic trioxide induced apoptosis [21]. Normal metabolism requires constant and rapid replenishment of GSH. Low level of GSH in experimental rats which were treated rats showed in this study may be due to over utilization of this antioxidant enzyme. Chen et al [22] reported decreased content of GSH in myocardial tissue damage during arsenic. Combination therapy with astaxanthin increased the level of GSH as compared with arsenic treated group. This may be due to the stabilization of sulphydryl group by astaxanthin. Up regulated GSH content is protection against reactive oxygen species (ROS) and free radical generation. The antioxidant activity of several carotenoids has been investigated by Palozza et al [23] and our results agreement with these findings. The hydroxyl and keto groups on each ring system of keto carotenoid compound astaxanthin give itself as a good antioxidant and this molecule have the capability to spans across the lipid bilayer of plasma membrane [24]. Astaxanthin intake evidently increased the superoxide scavenging activity and lowered the level of superoxide and hydroperoxides [25]. Carotenoids are believed to have therapeutic benefit in treating cardiovascular disease because of their antioxidant properties.

Lipid peroxidation is a primary measure of oxidative damage in tissue. In arsenic treated group the amount of malondialdehyde (MDA) was increased due to free radical formation. Membrane phospholipids decay was occur due to free radical generation. In arsenic treated group, the level of lipid peroxidation end product MDA was elevated compared to control rats. In arsenic treated group level of reactive oxygen species (ROS) was increased which may react with membrane phospholipids and initiates oxidation. Thioobarbituric acid reactive substances (TBARS) measured as a marker of lipid per oxidation in vivo[26]. Lipid per oxidation increases MDA value and decreases GSH level, because lipid hydro peroxides reduction in biological system occurs via through the expenses of GSH as antioxidant. Conjoint therapy with astaxanthin in experimental rats had shown a significant in the reduction of MDA level in heart tissue. In 2010 Guerin et al [3] also observed the capability of astaxanthin as a potent quencher of singlet oxygen and an effective inhibitor of lipid peroxidation. The keto group activates the hydroxyl group and hence facilitates hydrogen transfer to the peroxy radicals. Astaxanthin act as a strong antioxidant by donating the electrons and creating with free radicals to convert them to more stable product and terminates free radical chain reaction in a wide variety of living organisms. Mc Nulty et al [27] proposed that carotenoids exert differing effects based on their interactions with membranes. Astaxanthin improves anti tumor immune responses by inhibiting lipid peroxidation induced by stress [28]. Oxidative stress plays major role in cardiotoxicity by generation of lipid peroxidation and altered antioxidant enzyme activities [29].

In histopathological analysis shown abnormal ultra structural changes during arsenic intoxicated group compared to normal control. Histological examination reveals that arsenic trioxide treatment caused fibre swelling, and haemorrhages in the cardiac tissue. Arsenic trioxide increases the level of oxygen reactive species which induced apoptosis of the cell as also evident by the Soile et al [30]. In combined group no such morphological changes compared with As$_2$O$_3$ treated group was shown. A marked disorganization was caused arsenic such as fibre swelling, necrosis and haemorrhage in the cardiac tissue. In astaxanthin treated group there was no such clinical alterations such as necrosis and swelling. AS$_2$O$_3$ administration showed a histopathologic evidence of cardiomyopathy [31]. Arsenic induces cytotoxic effects in cardiomyocytes that are mediated through ROS leading to apoptosis via caspase-3 signaling. Combined supplementation of astaxanthin along with arsenic maintained the normal physiology of the myocardium.

**IV. Conclusion**

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In conclusion, we would like to mention that arsenic treatment caused severe oxidative impairment in the cardiac tissue of the experimental animals and that could be prevented by the cotreatment with astaxanthin. The overall protective role of astaxanthin is probably due to its free radical scavenging and thereby diminishing the arsenic burden in the heart tissue. However, further investigations are necessary to identify the responsible mechanism behind protective efficacy of this keto carotenoid astaxanthin.

References