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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2022.191.200

Research Article
Astaxanthin Attenuates D-Galactosamine-Induced Pancreatic Injury by Activating Antioxidant Enzymes and Inhibiting VEGF-C Gene Expression

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Abstract

Background and Objective: Astaxanthin (3,3′-dihydroxy-β-β-carotene-4,4′-dione) is a carotenoid, commonly found in marine environments has been reported to possess versatile biological properties including anti-inflammatory and antioxidant. In this study, the pancreatic protective effect of astaxanthin was investigated in D-Galactosamine-induced pancreas injury in rats. **Materials and Methods:** In this experimental study, MTT assay was used to determine cytotoxic effects of the Astaxanthin on pnc1 cells. A total of 30 adult albino rats divided into 5 groups, six rats in each. Group I was given an equal amount of distilled water, group II was received 400 mg kg^{-1} b.wt. D-galactosamine on 15th day, groups III-V were treated with astaxanthin (50 and 100 mg kg^{-1}) and/or silymarin (50 mg kg^{-1}) for 14 days + 400 mg kg^{-1} b.wt. D-galactosamine on the 15th day, respectively. **Results:** IC_{50} of Astaxanthin against the pnc1 cell line was 92.9 μg mL^{-1}. The daily oral administration of astaxanthin (50 and 100 mg kg^{-1}) as well as silymarin (50 mg kg^{-1}) for 14 days to rats treated with D-galactosamine resulted in a significant improvement in plasma AST, ALT, ALP as well as pancreatic TNF-α, IL-1β, IL-10, NO and VEGF-C gene expression. On the other hand, inducible oral administration of astaxanthin increased the activity of pancreatic GSH, SOD, GPx, GR, CAT and the level of TBARs in D-galactosamine-treated pancreatic of rats. Furthermore, Astaxanthin almost normalized these effects in pancreatic tissue histoarchitecture and MRI examination. **Conclusion:** The obtained results showed that Astaxanthin protected experimental animals against D-galactosamine-induced pancreatic injury through activation of antioxidant enzymes and IL-10 and inhibition of VEGF-C activation.

Key words: Astaxanthin, d-galactosamine, pancreas, antioxidant enzyme and cytokine storm

Citation: Emara, A.A., M.H. Mohamed, E.S. Nada, N.A. hashem and E.S. Mahmoud *et al*., 2022. Astaxanthin attenuates D-galactosamine-induced pancreatic injury by activating antioxidant enzymes and inhibiting VEGF-C gene expression. Pak. J. Biol. Sci., 25: 191-200.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

D-Galactosamine is one of the most experimentally used drugs to study hepatotoxic effects in experimental animals due to clinical features resembling acute hepatitis[1,2]. It alters uridine diphosphate (UDP)-glucose and UDP-galactose biochemically, which leads to a loss of intracellular calcium homeostasis and impairs hepatocyte energy consumption[3]. These alterations have an impact on cell membranes and organelles as well as protein and nucleic acid synthesis, all of which contribute to the reported liver damage[4].

In vivo studies have shown that galactosamine possesses hepatotoxicity and induces the elevation of AST, ALT and ALP enzymes[5]. Additionally, i.p., administration of rats with galactosamine showed that a significant depletion of antioxidant enzymes SOD, CAT, GPx and GR[6] as well as an increase in levels of inflammatory mediators[7].

Astaxanthin is a carotenoid that occurs naturally in a wide range of living organisms and is classed as a xanthophyll[8]. It has a chemical structure that is comparable to the well-known carotenoid-carotene. Astaxanthin has been suggested to protect muscle cells from oxidative stress[9]. The potency of astaxanthin's strong antioxidant activity was explained by the presence of hydroxyl (OH) and ketone (C=O) moieties on each ionone ring as well as an expansion of the conjugated double bond system[10].

Anti-inflammatory[11], hepatoprotective[12] and cardioprotective[13] properties have been demonstrated for astaxanthin. Although astaxanthin has been shown to have antioxidant and hypolipidemic properties[14-17]. It is a powerful extinguisher of reactive nitrogen and oxygen species (ROS), particularly monovalent oxygen[18]. As a continuation of the ongoing investigation in the therapeutic potential of astaxanthin[19,20], this study presented a simple method for evaluating astaxanthin's preventive efficacy against D-Galactosamine-induced pancreatic damage in rats.

MATERIALS AND METHODS

Study area: The study was carried out at the Biochemistry Department, Faculty of Applied Medical Sciences, October 6 University, Egypt from September, 2020 to March, 2021).

Chemicals and reagents: Astaxanthin (92%) and D-galactosamine were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. All other chemicals used were of analytical grade.

Determination of astaxanthin cytotoxicity on pancreas carcinoma (pnc1) cell line: Cells were plated into a 24-well plate at a density of 1.0×10^6 cells/well. The pnc1 cells were exposed to astaxanthin at a concentration of 31.5, 63.5, 125, 250, 500 and 1000 µg ML^{-1} for 24 hrs. Cells free of particles were used as control cells throughout each assay.

MTT assay was used to determine the effect of astaxanthin on the viability of pnc1 cell lines. After exposure, the pnc1cells were cultured for 4 hrs with MTT (20 µL/well of 5 mg mL^{-1} stock). Mitochondrial dehydrogenases in living cells convert yellowish water-soluble MTT into water-insoluble formazan crystals that can be dissolved in DMSO. The medium was then withdrawn from each well and 200 µL of DMSO was added to dissolve the formazan crystals. The medium was removed from the suspension culture by centrifugation and then DMSO was added. A microplate reader was used to detect optical density at 570 nm after full mixing (Biotek, USA).

Animals: The experiments were carried out using male albino rats weighing 210±10 g was obtained from the animal house of the National Cancer Institute, Cairo University, Giza, Egypt. Animals were provided with a standard diet and water *ad-libitum*. They were kept under constant environmental conditions and observed daily throughout the experimental work. They were housed in polypropylene cages (47×34×20 cm) lined with husk, replaced every 24 hrs, under a 12:12 hrs light: dark cycle at around 22°C. All animal experiments were conducted according to the guidelines of the Ethics Committee of the Faculty of Applied Health Sciences Technology, October 6 University, Egypt (registration No. 20210115).

Treatments and groups: Hepatotoxicity and pancreatic damage were induced in rats by intraperitoneal injection of D-galactosamine (400 mg kg^{-1} b.wt.) in 1% tween 80 for one day (21).

Astaxanthin was brought into suspension in 1% tween 80 for intragastric intubation of rats.

Rats were divided into 5 groups, 6 animals each. Selection of the doses of the used test substances illustrated in Table 1.

After 15 days of treatment, blood samples were drawn from a retro-orbital vein and collected in heparin-containing tubes. The heparinized blood samples were centrifuged for 20 min at 1000×g. Fluitest® GPT (ALT) kit, Fluitest® GOT (AST) kit and Fluitest® ALP-kit was used to evaluate plasma levels of Glutamic-Oxaloacetic Transaminase (GOT), Glutamic-Pyruvate Transaminase (GPT) and Alkaline Phosphatase (ALP).

Table 1: Description of treatment groups

Groups	Group names	Treatment description
I	Normal control A	3 mL of distilled water, orally for 15 days
II	D-galactosamine	Subcutaneous injection of 400 mg kg^{-1} b.wt. D-galactosamine in 1% tween 80, on 15th day[21]
III	Astaxanthin+D-galactosamine	Rats were treated with Astaxanthin (50 mg kg^{-1}) suspended in 1% tween 80, orally) for 14 days (19).+ Subcutaneous injection of 400 mg kg^{-1} b.wt. D-galactosamine in 1% tween 80, on 15th day
IV	Astaxanthin+D-galactosamine	Rats were treated with Astaxanthin (100 mg kg^{-1}) suspended in 1% tween 80, orally) for 14 days+ Subcutaneous injection of 400 mg kg^{-1} b.wt. D-galactosamine in 1% tween 80, on 15th day
V	Silymarin+D-galactosamine	Rats were treated with silymarin (50 mg kg^{-1}, p.o.) suspended in 1% tween 80, orally) for 14 days[22] + Subcutaneous injection of 400 mg kg^{-1} b.wt. D-galactosamine in 1% tween 80, on 15th day

Pancreatic specimens: At the end of the experiment, cervical decapitation sacrificed rats from each group. The pancreatic tissue was collected after autopsies from each group and washed with ice-cold saline to remove the blood. The tissue was minced and homogenized in 3 mL of phosphate buffer (pH 7.2) and centrifuged (×3000 g for 10 min). The clear supernatant was used for the assessment of GSH[23] and TBARs[24] calorimetrically using the kit of Cayman Chemical Company (Ann Arbor, MI).

SOD activity was assayed by the method of tetrazolium salt for detection of superoxide radicals generated by red formazan dye reduction produced[25]. GPx activity was measured indirectly by a coupled reaction with GR[26]. GR activity was assayed[27], with some modifications, by measuring the oxidation of NADPH at 340 nm. The Catalase assay kit utilizes the peroxidative function of CAT for the determination of enzyme activity[28].

Estimation of cytokines

Pancreatic cytokines: TNF-α[29], interlinkin-1β (IL-1β)[30] and interleukin-10 (IL-10)[31] were detected using a UV microplate reader (Thermo Electric Corp., Shanghai, China) according to the manufacturer's instructions. Add acid stopped the reaction and a micro platform reader measured the absorption at 450 nm.

Quantitative real-time PCR: The total RNA extracted was extracted from the pancreas of the rats and portions of (10-15 μg) of the isolated RNA were subjected to quantitative PCR analysis in real-time, using Sepasol-RNA1Super according to instructions of the manufacturer. The two-step RT-PCR gene expression has been measured. The level of VEGF-C was quantified with the previously described quantitative real-time PCR[32]. The tests in a 50 mL single-plex reaction mixture were conducted. Conditions of reaction were a pre-incubation at 50°C in 2 min, followed by 10 min by 40 cycles of 95°C in 15 sec and 60°C in 1 min, respectively.

Primer sequences were VEGF-C: F5-AACGTGTCCAA GAAATCAGCC-3, R: 5-AGTCCTCTCCCGCAGTAATCC-3. The internal control used GAPDH -F: 5-CTCAACTACATGG TCTACATGTTCCA-3 and -R: 5 -CCATTCTCGGCCTTGA-CTGT-3′.

Histological assessment: For the histological study, the pancreas was cut into pieces and fixed in a 10% buffered formaldehyde solution[33]. The sections were then examined under the microscope for histopathological changes and photomicrographs were taken.

MRI protocol: Once placed on the handling platform, each mouse was fixed in a supine recumbence position and then introduced into the RF coil inside the MRI gantry. Many images and sequences are taken for all rats in all groups to evaluate and compare the results, including CORONAL T1, T2, SAGITAL T1, T2 and STAIR.

Statistical analysis: Data were analyzed using one-way analysis of variance (ANOVA), followed by the least significant difference test using the Statistical Package for Social Science (SPSS) version 18.0 for windows (SPSS, Inc., IBM, Chicago, Illinois, USA). The results were expressed as Mean±SD. Values of $p<0.05$ were considered statistically significant.

RESULTS

The effect of astaxanthin on pancreatic cancer cells and normal cells was determined by cultures of astaxanthin at different concentrations (31.25, 62.5, 125, 250, 500 and 1000 μg mL^{-1}). The negative control was dimethyl sulfoxide (DMSO, 0.1%). IC_{50} panc1cells = 92.9 μg mL^{-1}) after 48 hrs in Table 2.

Table 3 showed plasma AST, ALT and ALP levels. Intraperitoneally administration of D-galactosamine led to a significant increase in AST, ALT and ALP activity as compared to the normal control group ($p<0.05$), indicating acute liver injury. Treatment of animals with astaxanthin at 50 and 100 mg kg^{-1} b.wt. as well as silymarin (50 mg kg^{-1} b.wt.) significantly decreased the level of AST, ALT and ALP ($p<0.05$) as compared to the D-galactosamine treated group.

Table 2: IC_{50} of Astaxanthin cytotoxicity on pancreas carcinoma (panc1) cell line

Compounds	Conc. µg mL^{-1}	O.D			Mean O.D	ST.E	Viability (%)	Toxicity (%)	IC_{50}
Astaxanthin	DMSO, 0.1%	0.362	0.378	0.352	0.364	0.007572	100	0	
	1000	0.02	0.019	0.02	0.019667	0.000333	5.402930403	94.5970696	92.9
	500	0.02	0.023	0.021	0.021333	0.000882	5.860805861	94.13919414	
	250	0.022	0.019	0.025	0.022	0.001732	6.043956044	93.95604396	
	125	0.105	0.118	0.101	0.108	0.005132	29.67032967	70.32967033	
	62.5	0.214	0.236	0.245	0.231667	0.009207	63.64468864	36.35531136	
	31.25	0.359	0.371	0.36	0.363333	0.003844	99.81684982	0.183150183	

O.D: Optical density and S.T.E: Standard Error

Table 3: Levels of serum aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP) in normal and experimental groups of rats

Groups	AST (U L^{-1})	ALT (U L^{-1})	ALP (U L^{-1})
Normal control A	10.76±0.75^{a}	7.36±0.65^{a}	265.0±16.09^{a}
D-galactosamine	19.84±1.35^{c}	15.64±1.76^{b}	370.6±21.66^{d}
Astaxanthin (50 mg kg^{-1} b.wt.)	13.88±0.98^{b}	8.77±0.98^{a}	282.90±17.38^{c}
Astaxanthin (100 mg kg^{-1} b.wt.)	10.05±1.88^{a}	7.15±0.83^{a}	274.67±18.74^{b}
Silymarin (50 mg kg^{-1} b.wt.)	11.47±1.54^{a}	7.39±0.47^{a}	266.45±15.98^{b}

D-galactosamine was given intraperitoneally as a single dose of 400 mg kg^{-1} b.wt.) on the 15th day, It was given to all groups except the normal one. Astaxanthin (50 and 100 mg kg^{-1} b.wt.) and Silymarin (50 mg kg^{-1} b.wt.) were orally given daily for 14 days and the last dose of each drug was given 1 h before paraquat administration, values are given as Mean±SD for groups of six animals each, data followed by the same letter are not significantly different at $p \leq 0.05$

Table 4: Levels of pancreatic reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) and thiobarbituric reactive substances (TBARS) in normal and experimental groups of rats

Groups	GSH mg%	SOD	GPx	CAT	GR	TBARS (nmol mg^{-1} protein)
Normal control A	17.59±1.62^{b}	21.90±2.65^{b}	9.75±0.82^{b}	57.40±5.44^{b}	14.98±0.86^{b}	35.65±3.76^{a}
D-galactosamine	5.33±0.73^{a}	13.06±0.64^{a}	4.16±0.42^{a}	36.77±3.28^{a}	8.05±0.98^{a}	52.65±4.20^{c}
Astaxanthin (50 mg kg^{-1} b.wt.)	15.90±0.84^{b}	19.53±1.80^{b}	8.15±0.74^{b}	68.00±3.98^{c}	12.40±1.08^{b}	38.24±3.77^{b}
Astaxanthin (100 mg kg^{-1} b.wt.)	16.08±0.75^{b}	20.71±2.80^{b}	8.96±0.55^{b}	55.63±6.15^{b}	14.00±1.22^{b}	34.94±4.09^{a}
Silymarin (50 mg kg^{-1} b.wt.)	17.00±0.87^{b}	19.84±2.08^{b}	8.50±0.73^{b}	55.40±4.09^{b}	13.87±1.09^{b}	36.17±2.65^{a}

D-galactosamine was given intraperitoneally as a single dose of 400 mg kg^{-1} b.wt.) on the 15th day, It was given to all groups except the normal one. Astaxanthin (50 and 100 mg kg^{-1} b.wt.) and Silymarin (50 mg kg^{-1} b.wt.) were orally given daily for 14 days and the last dose of each drug was given 1 h before paraquat administration, values are given as Mean±SD for groups of six animals each, data followed by the same letter are not significantly different at $p \leq 0.05$, SOD: One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1min/mg protein; GPx: µg of GSH consumed/min mg protein; GR: nmol of NADPH oxidized/min mg protein; CAT: µmol of H2O2 utilized/min mg protein

Table 5: Levels of pancreatic tumour necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-10 (IL-10) and nitric oxide (NO) in normal and experimental groups of rats

Groups	TNF-α (pg g^{-1} tissue)	IL-1β (pg g^{-1} tissue)	IL-10 (pg g^{-1} tissue)	NO (pg g^{-1} tissue)
Normal control A	7.43±0.36^{a}	15.09±1.04^{a}	26.85±3.64^{c}	11.76±0.54^{a}
D-galactosamine	18.65±1.05^{b}	32.10±3.00^{b}	16.05±1.38^{a}	17.15±0.76^{b}
Astaxanthin (50 mg kg^{-1} b.wt.)	9.80±0.66^{a}	17.88±1.25^{a}	23.43±1.50^{b}	12.65±1.04^{a}
Astaxanthin (100 mg kg^{-1} b.wt.)	7.00±0.50^{a}	15.24±0.78^{a}	27.09±3.05^{c}	10.76±1.33^{a}
Silymarin (50 mg kg^{-1} b.wt.)	8.36±0.73^{a}	14.18±0.66^{a}	25.40±2.71^{c}	11.53±0.85^{a}

D-galactosamine was given intraperitoneally as a single dose of 400 mg kg^{-1} b.wt.) on the 15th day, It was given to all groups except the normal one. Astaxanthin (50 and 100 mg kg^{-1} b.wt.) and Silymarin (50 mg kg^{-1} b.wt.) were orally given daily for 14 days and the last dose of each drug was given 1 h before paraquat administration, values are given as Mean±SD for groups of six animals each, data followed by the same letter are not significantly different at $p \leq 0.05$

Table 4 showed a significant decrease in pancreatic of SOD, GR, GPx, CAT and GSH as well as a significant increase in TBARS levels ($p<0.05$) in rats treated with D-galactosamine compared to the control group. The administration of Astaxanthin at 50 and 100 mg kg^{-1} b.wt. as well as silymarin (50 mg kg^{-1} b.wt.) showed a significant increase in SOD, GR, GPx, CAT and GSH as well as a significant decrease in TBARs levels compared with the D-galactosamine, treated group of rats after 15 days ($p< 0.05$).

Table 5 showed a significantly ($p<0.05$) increased in pancreatic TNF-α, IL-1β and NO while a significant decrease in pancreatic IL-10 was observed in the D-galactosamine-treated rats as compared to the normal control group ($p<0.05$), indicating acute pancreatic damage. The administration of rats with astaxanthin at 50 and 100 mg kg^{-1} b.wt. as well as silymarin (50 mg kg^{-1} b.wt.) decreased the pancreatic TNF-α, IL-1β and NO in rats and increased-10 level as compared to the D-galactosamine-treated group.

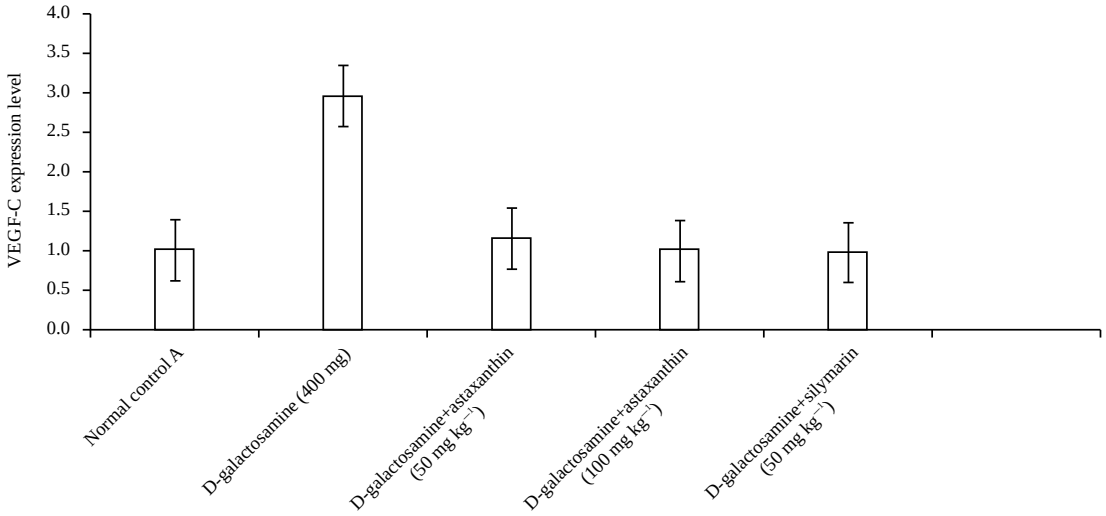

Fig. 1: Effect of astaxanthin (50 and 100 mg kg^{-1} b.wt.) and silymarin (50 mg kg^{-1} b.wt.) on levels of pancreatic vascular endothelial growth factor C (VEGF-C) gene expression in D-galactosamine-treated rats

A representative bar diagram of three independent experiments is presented

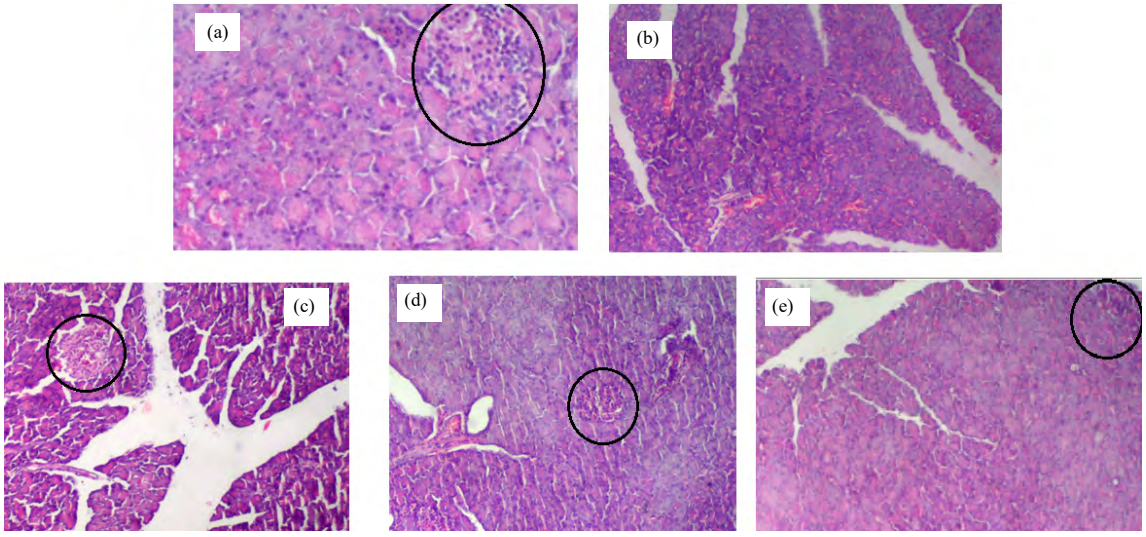

Fig. 2(a-e): Sections stained with hematoxylin and eosin (H and E; 400 X) histological examination of the pancreas of different groups compared to control group

(a) Group I: Normal control, (b) II: D-galactosamine (400 mg kg^{-1} b.wt.) group, (c) III: D-galactosamine+Astaxanthin (50 mg kg^{-1} b.wt.), (d) IV: D-galactosamine+Astaxanthin (100 mg kg^{-1} b.wt.) and (e) V: D-galactosamine+Silymarin (50 mg kg^{-1} b.wt.)

Figure 1 showed a significantly ($p<0.05$) increased in pancreatic VEGF-C gene expression was observed in the D-galactosamine-treated rats as compared to the normal control group ($p<0.05$), indicating acute pancreatic damage. The administration of rats with astaxanthin at 50 and 100 mg kg^{-1} b.wt. as well as silymarin (50 mg kg^{-1} b.wt.) decreased the pancreatic VEGF-C gene expression level as compared to the d-galactosamine-treated group.

Histopathological examination of pancreas sections of the normal group I showed regular acini and normal size of the B cell isle(the circle) x 400 H and E in Fig. 2a.

On the other hand, in the pancreatic tissue of D-galactosamine-treated control group II, histological examination showed an atrophic B cell islet. The blood vessels are congested X200H and E in Fig. 2b. Histopathological examination also showed good recovery of D-galactosamine-

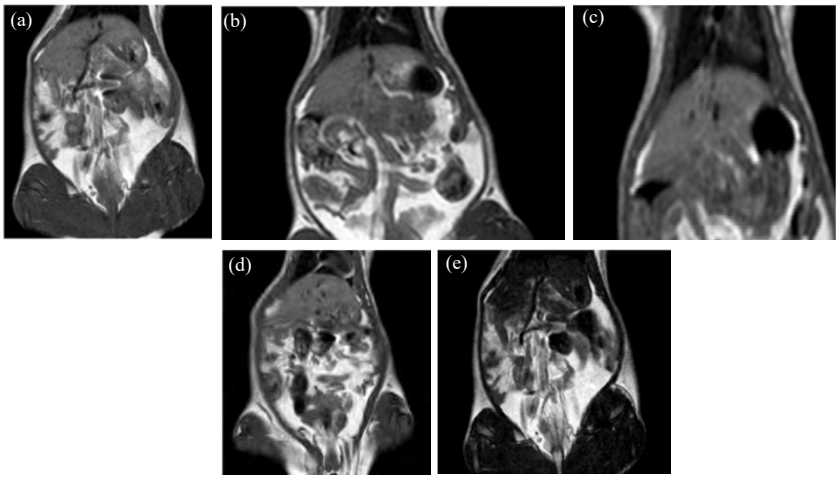

Fig. 3(a-e): MRI examination of the pancreas of different groups compared to control group

(a) Group I: Normal control, (b) II: D-galactosamine (400 mg kg^{-1} b.wt.) group, (c) III: D-galactosamine+Astaxanthin (50 mg kg^{-1} b.wt.), (d) IV: D-galactosamine+Astaxanthin (100 mg kg^{-1} b.wt.) and (e) V: D-galactosamine+Silymarin (50 mg kg^{-1} b.wt.)

induced pancreatic toxicity by administration of Astaxanthin (50 and 100 mg kg^{-1} b.wt.) as well as silymarin (50 mg kg^{-1} b.wt.) as compared to the D-galactosamine-treated group and showed almost the same records as Groups Fig. 2c-e.

MRI examination of the pancreas of the normal group I showed that the pancreatic parenchyma was regular, homogenous with normal echogenicity in Fig. 3a. Also, the pancreatic MRI examination of D-galactosamine-treated rats (II), showed pancreatic masses that appear as irregular, heterogeneous, with alternative intensity in Fig. 3b.

MRI examination also showed coarse texture and the abnormal focal lesion was improved in D-galactosamine-treated rats +Astaxanthin at 50 mg kg^{-1} b.wt. as compared with the D-galactosamine-treated rats (III) in Fig. 3c. In addition, pancreatic examination by MRI from D-galactosamine-treated rats treated with Astaxanthin 100 mg kg^{-1} b.wt. Group (IV) showed pancreatic repair and regeneration was regressed at different levels in Fig. 3d. In addition, all samples of D-galactosamine-treated rats showed moderate focal lesions were regressed by treatment with silymarin (50 mg kg^{-1} b.wt.) group (V) in Fig. 3e.

DISCUSSION

The current study found that astaxanthin has cytotoxic effects on the pancreas carcinoma (panc1) cell line. Astaxanthin has been shown to have cytotoxic and apoptotic effects on cancer cells in prior research[34]. It has recently been shown to have pro-apoptotic and anti-cancerous actions on an intestinal human colon adenocarcinoma cell line[35]. The findings also point to astaxanthin's potential role in pancreatic cancer treatment. Recent investigations have shown that astaxanthin has anticancer potential in cancer cells *in vitro* via mitochondrial-mediated apoptosis[36,37].

D-Galactosamine is a well-known hepatotoxin that has been studied extensively. Usually used to cause acute liver damage in rats. Hepatocytes' uridine pools are depleted by galactosamine metabolism, resulting in the transcriptional block. It also causes TNF-α transcription, oxidative stress and GSH depletion, all of which lead to mitochondrial malfunction and cell death[38]. The pathophysiology of GAL-induced hepatic damage is complicated by both oxidative and nitrosative stress[39].

In this study, the administration of D-Galactosamine significantly increased the levels of ALT, AST and ALP, which are indicative of failing liver function. The impairment of liver function has been seen by the raised levels of ALT, AST and ALP in the D-galactosamine treated animals.

Furthermore, oral administration of astaxanthin at 50 and 100 mg kg^{-1} b.wt. as well as silymarin (50 mg kg^{-1} b.wt.) provided significant protection against D-galactosamine-liver damage. Recent studies reported that astaxanthin could alleviate oxidative stress in vitro and Vivo. In human vascular endothelial cells, it would attenuate glucose fluctuation-caused oxidative stress and cell apoptosis[40] *in-vivo*, Astaxanthin was showed to significantly ameliorate hepatic injury by reducing ROS level and inhibiting inflammatory cytokines pathway[41].

Pancreatic cells are vulnerable to oxidative damage for two reasons: one, they have a weak antioxidant defence system and the other, they produce too much Reactive Oxygen Species (ROS) within the cell as a result of the

general public's daily exposure to harmful substances[42]. D-galactosamine is a poisonous substance that produces reactive oxygen species on its own. However, multiple investigations have found that D-galactosamine-induced oxidative stress is a widespread occurrence[3]. It may cause a large increase in liver enzymes and lipid peroxidation as well as a considerable elevation in MDA and drop-in antioxidant enzyme activity in pancreatic tissue when exposed to it[43].

Nitric oxide, a compound that plays a complex function in both oxidative stress and cell death responses, is produced in response to oxidative stress. In this investigation, the activity of TNF-α, IL-1β, IL-10 and NO was measured in plasma and the oxidation end product nitrite was dramatically elevated in tandem with the gene expression of the VEGF-C enzyme in the D-galactosamine group. One of the most important effects of the NOS-2 enzyme is that when it is activated by higher amounts of TNF-α, it creates nitric oxide, which stimulates the synthesis of more TNF-α, resulting in inflammatory damage[44]. Furthermore, nitric oxide is thought to play a dual role in apoptosis acting as both proapoptotic and anti-apoptotic mediators depending on various cellular conditions and cell types[45]. Some studies have shown that this inducible isoenzyme in certain cell types contributes to cell death by increasing caspase 3 activity due to increased cytokine levels such as TNF- α[46].

The GPx, SOD, CAT, GR and GSH levels in the control and D-galactosamine groups were identical in this investigation, indicating that the GSH pool has remained intact. In addition to GSH's antioxidant activity, the antioxidant enzymes SOD, GPx, GR and CAT collaborate to prevent oxidation of proteins, lipids and DNA by eliminating reactive oxygen species from the cell[47].

Specifically, SOD reduces superoxide into hydrogen peroxide, which is further reduced to water by the action of catalase and glutathione peroxidase[48]. It is noteworthy that within the present experimental conditions gene expression of SOD, GPx, GR and CAT decreased significantly in pancreatic tissue. It might be expected that under the present experimental conditions, the responses of these three parameters would be increased in parallel, however, under D-galactosamine toxicity the expected depletion of these antioxidant enzymes was seen. Differential response of these enzymes may be dependent on the dictating cellular needs in the fight against increased levels of reactive oxygen species in induced oxidative stress states[48].

The presence of the hydroxyl and ketone endings on each ionone ring of Astaxanthin, explains some unique features of its structure, such as its ability to be esterified, its high anti-oxidant activity[49-51] and a more polar configuration than other carotenoids[51]. Free astaxanthin is extremely sensitive to oxidation, however its esterified form as found in nature in the *Haematococcus pluvialis* (*H. pluvialis*) microalga[52], is stable and displays higher bioavailability and potency[53,54].

The obtained data in the present study demonstrated that astaxanthin considered a potent antioxidant agent when given simultaneously with isoproterenol since it could produce a marked increase in pancreatic SOD, GPx, CAT and GSH levels towards the normal values. Many authors have reported the effective antioxidant action of astaxanthin. Astaxanthin has unique chemical properties based on its molecular structure[9,19]. As illustrated in Fig. 1 the presence of hydroxyl (OH) and ketone (C=O) moieties on each ionone ring along with an extension of the conjugated double bond system explained the potency of astaxanthin with higher antioxidant activity[20]. The two groups most prominent antioxidant activities of astaxanthin are quenching of singlet oxygen and inhibition of lipid peroxidation[19, 20]. In the current model, Astaxanthin provides cell membranes with a potent protector against free radicals or another oxidative attack. Experimental studies confirm that this nutrient has a large capacity to neutralize free radicals or other oxidant activity in the nonpolar (hydrophobic) zones of phospholipid aggregates as well as along their polar(hydrophilic) boundary zones[55].

The current research explored the antidote effect of astaxanthin and silymarin against D-galactosamine -induced pancreatic toxicity in the way of oxidative stress and related pathways, such as TNF-α, IL-1β, IL-10 and NO etc. Extensive reports showed that ROS was closely related to the cytokines pathway[56]. These findings are consistent with our results. Mitani *et al.*[57] demonstrated that induction of HO-1 expression could inhibit inflammatory reaction through decreasing oxidative stress in rats intestines. Preventing TNF-α, IL-1β, IL-10 and NO elevation and mitochondrial pathway could ameliorate high ROS liberated by liver damage[58]. All the biochemical parameters especially, VEGF-C highlighted the role of oxidative stress in the mechanism by which astaxanthin ameliorated D-galactosamine toxicity in pancreatic cells.

According to histological studies, Astaxanthin has a pancreatic and hepato-protective effect. Because pancreatic proliferation is an early event in toxicity-related changes, the attenuation of pancreatic injury and fibrosis in rats by Astaxanthin could be associated with a reduction in the inflammatory response. To the best of our knowledge, the prophylactic effect of astaxanthin against D-galactosamine-induced pancreatic and hepato-protective injury has never been reported and this study may be the first of its kind.

CONCLUSION

The current study found that astaxanthin has potent protective activity against D-galactosamine-induced pancreatic toxicity by normalizing the levels of oxidative stress biomarkers and inflammatory mediator gene expression. Also, current results demonstrated that Astaxanthin prevents the liberation of ROS for the damaged liver cell by D-galactosamine administration of D-galactosamine by inhibits the pancreatic expression of TNF-α, IL-1β, IL-10 and NO as well as induces the production of pancreatic antioxidant enzymes.

SIGNIFICANCE STATEMENT

This study discovers the protective activity of astaxanthin that can be beneficial for the treatment of the pancreatic injury. This study will help the researcher to uncover the critical areas that focus on evaluate of astaxanthin as a promising new agent in the treatment of pancreatic toxicity that many researchers were not able to explore. Thus, a new theory to explain the correlation between pancreatic injury and cytokine signalling may be arrived at.

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