

# Astaxanthin alleviates oxidative damage in acute pancreatitis via direct antioxidant mechanisms

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## ABSTRACT

**Background/Aims:** Astaxanthin (ATX) is a naturally occurring carotenoid and a potent antioxidant. Various anti-inflammatory effects of ATX have been examined. We aimed to investigate the protective effect of ATX and its mechanism in a cerulein-induced acute pancreatitis rat model.

**Materials and Methods:** The rats were randomized into 2 main groups as control (C) and acute pancreatitis group (AP). AP group was subsequently divided into subgroups as AP+vehicle (AP), AP+ATX, and ATX+peroxisome proliferator-activated receptor-alpha antagonist GW6471 (ATX+GW) groups. To induce AP, the rats were administered cerulein (50 µg/kg, intraperitoneally [ip]) at 1 hour intervals, whereas the C group received saline. The AP group was treated with vehicle olive oil, ATX 40 mg/kg/orally, or GW6471 and ATX (GW1 mg/kg/ip; ATX; 40 mg/kg/peroral). Treatments were administered after the 1st cerulein injection. At the 7<sup>th</sup> hour after the final injection, the rats were killed and the pancreatic tissue was used for the determination of malondialdehyde (MDA), glutathione (GSH), and myeloperoxidase (MPO) activities and luminol-lucigenin chemiluminescence levels. Serum amylase, lipase, and histopathological analyses were performed.

**Results:** Elevated serum lipase and amylase levels in the vehicle-treated AP group ( $p < 0.01$ ) decreased in the ATX and ATX+GW groups ( $p < 0.05$ ). In the AP groups, GSH was reduced and MDA, MPO, luminol, and lucigenin levels were increased ( $p < 0.05-0.001$ ). ATX reversed these changes ( $p < 0.05-0.001$ ). The vehicle-treated group revealed significant severe cytoplasmic degeneration and vacuolization, whereas ATX ameliorated these destructions. GW6471 did not abolish the positive effects of ATX biochemically or histologically.

**Conclusion:** ATX has a potent protective effect on AP via its radical scavenging and antioxidant properties. Therefore, we believe that ATX may have therapeutic potential.

**Keywords:** Pancreatitis, inflammation, astaxanthin, free radical scavengers

## INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease characterized by a sudden inflammatory attack of the pancreas. Acinar damage owing to the autodigestive processes stimulates an inflammatory response in the parenchyma. In most cases, it is generally mild and can also be accompanied by interstitial pancreatic edema (1).

In the pathophysiology of AP, the key role of oxidative stress is well established. Free oxygen radicals induce acinar cell damage and trigger the inflammatory process, which leads to pancreatic edema and inflammatory cell accumulation (2). Previous experimental studies and clinical trials report that oxygen-derived metabolites and lip-

id peroxidation products are increased in the early phase of AP, and their levels correlate with the severity of the disease. Thus, improving the antioxidant status could result in a better clinical consequence in patients (3, 4). In addition, decrease in the plasma level of the antioxidant glutathione (GSH) is inversely proportional to the severity of disease (5).

Peroxisome proliferator-activated receptors (PPARs) are well-defined nuclear receptors that regulate the expression of genes in various biological pathways, such as cellular metabolic pathways, inflammation, differentiation, and proliferation. The alpha, beta, and gamma subtypes of the receptor have different metabolic functions (6, 7).

This study was presented at the 44<sup>th</sup> National Physiology Congress, Free Radical Scavenging Activity of Astaxanthin in Experimental Acute Pancreatitis Induced by Serulein, 01 - 04 November 2018, Antalya, Turkey.

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PPAR- $\alpha$  is known to be a general modulator of inflammation and was 1<sup>st</sup> reported by Devchand et al. (6). In a recent study, endogenous PPAR- $\alpha$  ligands reduced the severity of pancreatic damage caused by AP induced by cerulein administration (8). Similarly, an agonist of PPAR- $\alpha$  was shown to reduce the pancreatic damage in a cerulein-induced pancreatitis model (9).

Several antioxidants neutralizing the harmful oxygen radicals act as direct scavengers, and others that regulate the levels of antioxidant enzymes act indirectly. Meta-analysis of experimental studies suggests that antioxidant therapy can be beneficial in AP (10, 11). However, the clinical benefits of antioxidants are still controversial owing to the limited number of the patients studied (12).

Astaxanthin (ATX) is a naturally occurring carotenoid in the marine environment. Studies have reported about its powerful antioxidant and free radical scavenger effects (13, 14), and it is also known to be a PPAR- $\alpha$  receptor agonist (15).

The purpose of this study was to evaluate the protective effect and underlying mechanism of orogastric administration of ATX during the induction process of the disease in a cerulein-induced rat model.

## MATERIALS AND METHODS

### Animals

Female Sprague-Dawley rats (6–8 months old) obtained from the Marmara University Experimental Animals Research and Implementation Centre (Istanbul, Turkey) were kept under controlled conditions of humidity (65%–70%), temperature (22°C±2°C), and under standard light/dark (12 hour/12 hour) cycles. The rats were fed with standard rat pellets and water *ad libitum*. In compliance with the Turkish law on the use of animals in experiments, all the experimental protocols were approved by the Marmara University Animal Care and Use

### MAIN POINTS

- Astaxanthin, a potent antioxidant significantly reduces oxidative stress and protects from pancreatic damage.
- Inhibition of PPAR- $\alpha$  does not block the effect of ATX in cerulein-induced AP.
- The antioxidant effects of astaxanthin are related to its scavenging activity and its increasing effect on antioxidant defense enzyme.
- Astaxanthin can be used as a valuable therapeutic approach in AP.

Committee (76. 2017.mar, and the principles and guidelines developed by the New York Academy of Sciences were followed.

### Experimental Design

The rats were divided randomly as control (C) and AP groups. The AP group was subsequently divided into 3 subgroups as vehicle-treated (AP), ATX- (Merck KgaA, Darmstadt, Germany) treated (ATX), and PPAR- $\alpha$  antagonist GW6471- (Merck KgaA, Darmstadt, Germany) and ATX-treated (ATX+GW) groups.

To induce AP, cerulein (Sigma-Aldrich Co. LLC, Germany) was injected twice at 1 hour intervals (n=18), whereas the C group (n=6) received saline. After the 1<sup>st</sup> cerulein injection, the AP group was treated with vehicle olive oil, ATX group was administered ATX, and ATX+GW group was treated with GW6471 and ATX. The antagonist GW6471 was applied 10 minutes before the ATX treatment (Figure 1). Lipid-based formulations might enhance the oral bioavailability of ATX; therefore, an oil-based formulation was used in this study.

Cerulein was dissolved in saline (50  $\mu$ g/kg) and intraperitoneally (ip) injected. ATX mixed in olive oil was administered via orogastric gavage (40 mg/kg) just after the 1<sup>st</sup> injection. GW6471, an antagonist of PPAR- $\alpha$ , was dissolved in saline and injected once (1 mg/kg/ip).

At the 7<sup>th</sup> hour after the final injection, the rats were killed by cardiac puncture under anesthesia by thiopental sodium (50 mg/kg/ip). The pancreatic tissue was removed, and half of the pancreatic tissue samples were fixed in 10% formaldehyde for histopathological analysis. The remaining tissues were stored at –80°C for measurement of the pancreatic malondialdehyde (MDA), GSH, myeloperoxidase (MPO) activities, and luminol and lucigenin chemiluminescence (CL) levels. Serum samples were collected for amylase and lipase measurements.

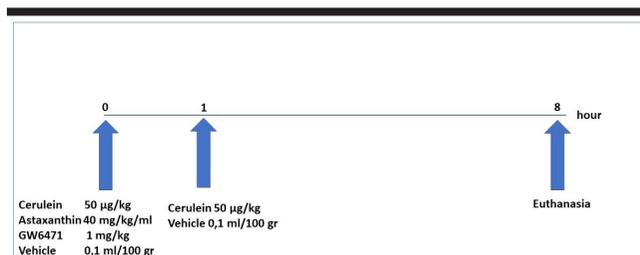


Figure 1. Experimental protocol. GW6471 applied 10 minutes before the astaxanthin treatment.

## Biochemical Analyses

### Serum amylase and lipase

Blood was collected via cardiac puncture and centrifuged at 3,000 rpm for 10 minutes at 4°C. The serum amylase and lipase levels were determined using enzyme-linked immunosorbent assay kits (eLabsience, San Diego, CA, USA) in accordance with the manufacturer's instructions and guidelines.

### MDA and GSH levels

MDA levels, as a marker of lipid peroxidation, were determined in the pancreatic tissues by monitoring thiobarbituric-acid-reactive substance formation, which has a maximum absorbance at 532 nm as previously described (16). The pancreatic samples were homogenized in 10 volumes of ice-cold 10% trichloroacetic acid and centrifuged (3,000 rpm for 15 minutes at 4°C). The supernatant was discarded and recentrifuged at 15,000 rpm at 4°C for 8 minutes. Lipid peroxide levels were expressed as MDA equivalents as nmol MDA/g tissue (16). GSH was measured using a spectrophotometric method via modification of the Ellman procedure (17). Briefly, after centrifugation at 10,000 rpm for 10 minutes, 0.5 mL of the supernatant was added to 2 mL of 0.3 mol/L of Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O solution, and then 0.2 mL solution of dithio-bisnitrobenzoate (0.4 mg/mL of 1% sodium citrate) was added to the mixture, and the absorbance at 412 nm was tested immediately after mixing. GSH levels were calculated using an extinction coefficient of 1.36×10<sup>4</sup> /M/cm. Results were expressed as GSH μmol/g tissue.

### MPO

MPO, a heme protein expressed by phagocytic cells, is found predominantly in the azurophilic granules. Tissue MPO activity is used to predict the tissue polymorphonuclear leukocytes (PMNs) accumulation, especially in the inflamed tissues, and correlates with the number of PMNs determined histologically. MPO activity was measured in 0.2–0.3 g of the pancreatic tissue samples. The pancreatic tissue was homogenized in 10 volumes of cold potassium phosphate buffer (pH 6.0, 50 mM of K<sub>2</sub>HPO<sub>4</sub>) containing hexadecyltrimethylammonium bromide (HET-AB; 0.5% w/v). The homogenate was centrifuged (10,000 rpm for 10 min at 4°C), and the supernatant was discarded. The pellet was homogenized with an equivalent volume of 50 mM K<sub>2</sub>HPO<sub>4</sub> containing 0.5% w/v HETAB and 10 mM ethylenediamine tetraacetate. MPO activity was assessed by measuring the H<sub>2</sub>O<sub>2</sub>-dependent oxidation of o-dianisidine-2 HCl. A single unit of enzyme activity was set as the amount of MPO that caused a change in the

absorbance tested at 460 nm for 3 minutes. MPO activity was expressed as U/g tissue (18).

### CL assay

We used the CL assay to determine the existence of reactive oxygen species (ROS) in the pancreatic tissue. It is a direct method to measure ROS where luminol and lucigenin can be used as enhancers. First, the pancreatic tissue samples were added to the tubes, including hydroxyethyl piperazineethanesulfonic acid (20 mM; pH 7.2) solution in phosphate-buffered saline (PBS; 0.5 M). After the addition of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) or lucigenin (bis-N-methylacridiniumnitrate) probes (final concentration: 0.2 mM), CL levels were tested as markers of ROS formation via a Junior LB 9509 luminometer (EG&G; Berthold, Germany). Lucigenin is sensitive to superoxide anion, whereas luminol is sensitive to peroxy-nitrite, hydrogen peroxide, hydroxyl radical, hypochlorite, and lipid peroxy radicals. Measurements were made at 1-minute intervals for 5 minutes. The results were calculated as the area under the curve and were adjusted for the wet tissue weight. All the results were expressed as relative light unit/mg tissue (rlu/mg) (19).

### Histopathological Analysis

For light microscopic evaluations, the pancreatic tissue samples were fixed in 10% neutral buffered formalin for 48 hours, dehydrated in ascending alcohol series (70%, 90%, 96%, and 100%), cleared with xylene, and then embedded in paraffin. Paraffin sections of 4-μm thickness were cut by rotary microtome (Leica RM2125RT, Wetzlar, Deutschland) and stained with hematoxylin and eosin dye for histopathological evaluations. The sections were analyzed and photographed through a light microscope (Olympus BX51, Tokyo, Japan), which was attached to a digital camera (Olympus DP72, Tokyo, Japan). Each section was evaluated histopathologically using the modified technique according to the following criteria: vascular congestion, vacuolization in acinar cells, interstitial edema, dilated ducts, leukocyte infiltration, and Langerhans islets disorders (Moreno) and scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) (20).

### Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by the post hoc Tukey's test and Student's t-test, and non-parametric histopathological data were analyzed by the Kruskal-Wallis and post hoc Dunn's using GraphPad Prism 6.0. Differences were considered significant if p<0.05. Values were expressed as mean±standard error of mean.

## RESULTS

### Effect of ATX on Serum Amylase and Lipase Levels

Serum lipase and amylase levels were elevated in the vehicle-treated group compared with the control group ( $p < 0.01$ ). Lipase and amylase levels were decreased in the ATX group compared with the vehicle-treated group ( $p < 0.05$ ), and this decrease stayed constant in the ATX+GW group ( $p < 0.05$ ) (Figure 2).

### Effect of ATX on MPO Activity and MDA Level

In the vehicle-treated group, MPO activity and MDA level increased significantly compared with those in the control group ( $p < 0.001$  and  $p < 0.01$ , respectively, Figure 3A-B). Elevated MPO activity and MDA levels were reduced by ATX treatment ( $p < 0.01$ ), and reduced levels were maintained in the ATX+GW group ( $p < 0.05$ ).

### Effect of ATX on GSH level

GSH levels decreased significantly in the vehicle-treated group compared with the control group ( $p < 0.001$ ). Decreased GSH level was elevated by the treatment of ATX ( $p < 0.01$ ), and significant increase in the levels with ATX

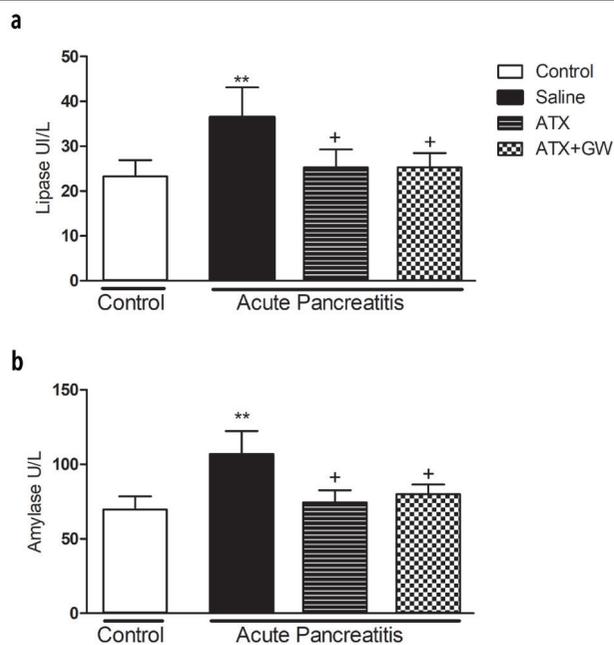
treatment was maintained in the ATX+GW group ( $p < 0.5$ ) (Figure 3c).

### Effect of ATX on CL values

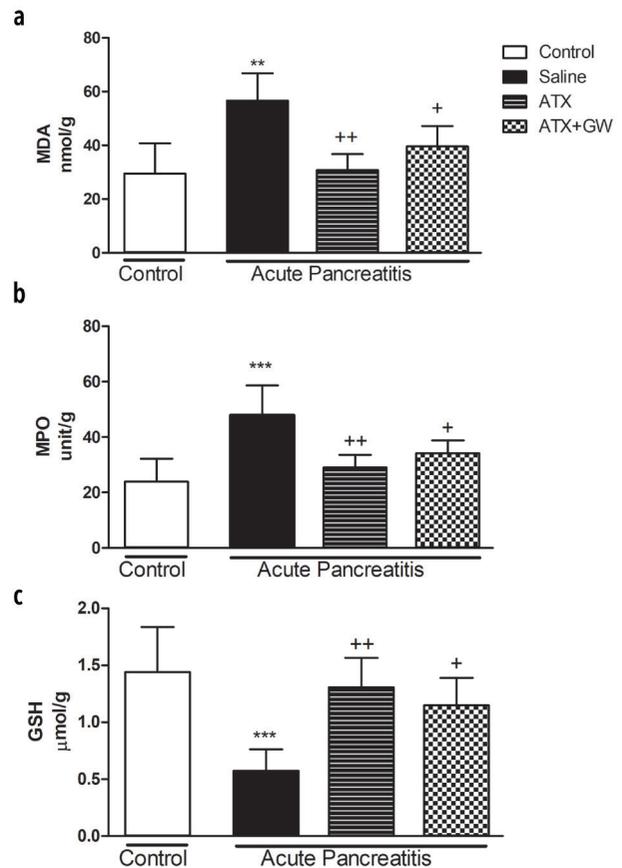
Both luminol and lucigenin levels were elevated in the saline-treated group compared with the control group ( $p < 0.5$  and  $p < 0.01$ , respectively; Figure 4). The elevated levels were significantly abolished by ATX treatment ( $p < 0.5$ ) with or without GW6471 ( $p < 0.5$  and  $p < 0.01$ , respectively).

### Histological Analyses

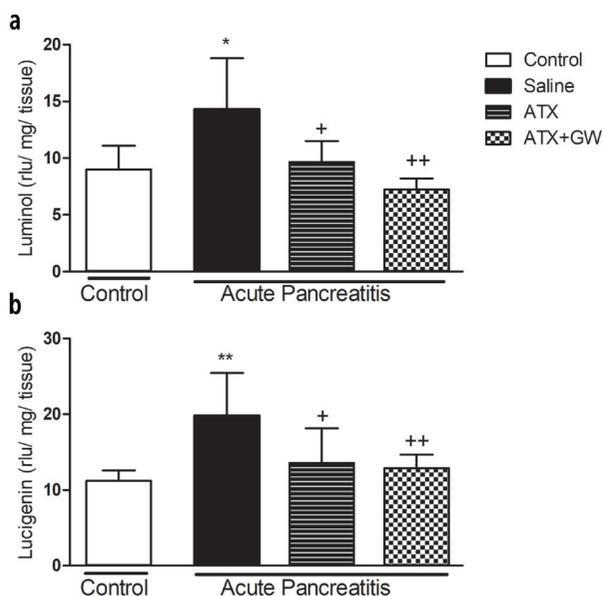
Well-organized acinar structures in the parenchymal tissue with regular morphology were observed in the histological examination of the control group (Figure 5A). The vehicle-treated group revealed significantly severe cytoplasmic degeneration and vacuolization, vascular con-



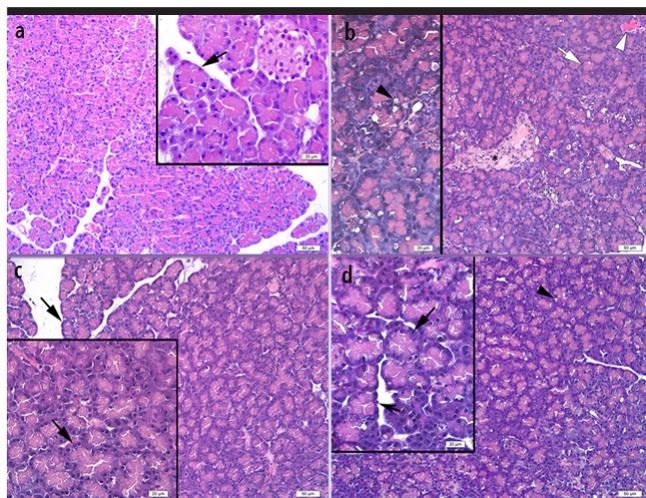
**Figure 2. a, b.** Lipase and amylase levels in sera. Lipase (a) and amylase (b) levels in the pancreatic tissues of the control and vehicle-treated groups, astaxanthin (ATX) and ATX+GW-treated acute pancreatitis groups ( $n=6$ /group). Data are expressed as mean  $\pm$  standard error of mean;  $n=6$  rats/group; \*\* $p < 0.01$  vs. control group; + $p < 0.05$  vs. vehicle-treated group.



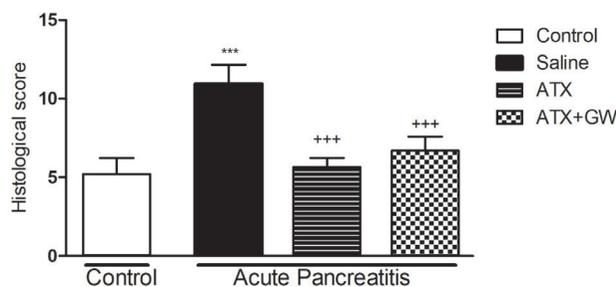
**Figure 3. a-c.** Pancreatic tissue, malondialdehyde, myeloperoxidase activity, and glutathione levels. Data are expressed as mean  $\pm$  standard error of mean;  $n=6$  rats/group; \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control group; + $p < 0.05$ , ++ $p < 0.01$  vs. vehicle-treated group.



**Figure 4. a, b.** Pancreatic luminol-enhanced (a)- and lucigenin-enhanced (b) tissue chemiluminescence levels. Data are expressed as mean±standard error of mean; n=6 rats/group; \*p<0.05, \*\*p<0.01 vs. control group; +p<0.05, ++p<0.01 vs. vehicle-treated group.



**Figure 5. a, b.** Representative photomicrographs of experimental groups. Control (a), vehicle (b), astaxanthin (ATX) (c), ATX+GW (d) groups. (a): Acinar structures have regular morphology (arrow). (b): Severe vacuolization in acinar cell (arrowhead), acinar structure with irregular morphology (white arrow), severe fibrosis (asterisk), inflammatory cell infiltration (white asterisk), severe vascular congestion (white arrowhead). (c): Acinar structure with regular morphology (arrow). (d): Acinar structure with regular morphology (arrow), slight inflammatory cell infiltration (notched-arrow), slightly vacuolization in acinar cell (arrowhead).



**Figure 6.** Microscopic score in the pancreatic tissues of experimental groups. Data are expressed as mean±standard error of mean; n=6 rats/group; \*\*\*p<0.001 vs. control group; +++p<0.001 vs. vehicle-treated group.

gestion, and inflammatory cell infiltration compared with those in the control group (Figure 5B). ATX treatment alleviated these destructions (Figure 5C-D).

Concomitant with the improvements examined histologically, the histological score was significant in all groups (p<0.001, Figure 6). In the vehicle-treated group, the histological score was increased significantly compared with that of the control group (p<0.001). The elevated score was reduced by ATX treatment, whereas the reduction in ATX treatment was constant in the ATX+GW group (p<0.001).

**DISCUSSION**

In this study, the effect of ATX on AP and its underlying mechanism of action were evaluated by means of a strong PPAR-α antagonist GW6471.

Oxidative stress occurs because of increased production of ROS and diminished antioxidant activity. The role of oxidative stress in inflammation has been reported in many studies (3, 4). Free oxygen radicals also play a role in the activation of AP and neutrophils and in the emergence of the inflammatory response (21). Once ROS was implicated in the pathophysiology of AP, several studies reported the ameliorative effect of many plant-derived natural products on the oxidative stress in AP (10, 22). In normal circumstances, ROS levels are balanced via the antioxidant defense system, which includes enzymatic and nonenzymatic antioxidants. These antioxidants neutralize the harmful free radicals and non-radicals. The imbalance between the oxidants and antioxidants causes structural damages, such as lipid peroxidation, which leads to the inflammatory process (4, 5). ATX has a potent capacity for scavenging ROS owing to the presence

of 2 oxygenated groups in its molecular structure (13, 14). The biological activity of ATX was greater than that of other antioxidants because of its transmembranous nature (23) therefore, we used ATX in oil in this study.

Our study confirmed the implication of reactive species at an early stage of cerulein-induced AP in rats and the ameliorative effect of ATX. In our study, parallel to the literature, MDA levels (24) and MPO activity (10, 5) were increased in the vehicle-treated group, whereas GSH levels decreased (24-26) in the AP group. In our study, MPO levels were decreased in the ATX group, which is consistent with a study by Zahng et al. (10). We also investigated MDA level, which is a sensitive marker of inflammation, in our groups. Multiple reports have revealed that ATX reverses the increased levels of MDA in inflammation (26, 27). The oxidative damage in the pancreatic tissue is the result of not only an increase in the ROS but also a decrease in the antioxidant capacity (25). Therefore, we investigated GSH, a tripeptide thiol and an important endogenous antioxidant defense molecule. Its intracellular concentration is an indicator of oxidative stress and holds a crucial part in the detoxification of ROS in the pancreas. Our data revealed that AP induced a decrease in the pancreatic GSH level, confirming the association of stress with oxidative damage, which is reversed by ATX. Our findings are consistent with those of previous studies, showing that ATX inhibits GSH depletion and increases GSH levels (25, 28).

CL is an assay for ROS. The luminol probe use of this technique detects  $\text{OH}^-$ ,  $\text{H}_2\text{O}_2$ , hypochlorite, peroxyxynitrite, and lipid peroxy radicals, and lucigenin is specific for superoxide radicals. In conformity with the previous studies, our results showed an increased pancreatic production of ROS. ATX diminished the increased luminol and lucigenin levels in AP just like in other inflammatory diseases (14). In our study, decreases in the serum enzyme levels and in the histological score were consistent and supported the protective effects of ATX.

PPAR- $\alpha$  is a member of the nuclear receptor superfamily of ligand-dependent transcription factors related to retinoid, steroid, and thyroid hormone receptors. PPAR- $\alpha$ , which is one of the subtype of PPAR family, is a nuclear receptor with wide-ranging effects on genes involved in fatty acid and lipoprotein metabolism and inflammation and highly expressed in the pancreas (29). A previous study revealed that different PPAR- $\alpha$  agonists suppressed inflammation by decreasing pro-inflammatory cytokines (30) and inflammatory mRNA expression (31)

and reduced pancreatic damage. In this study, we investigated the effect of ATX, which is suggested to be a PPAR- $\alpha$  agonist, on the cerulein-induced model of AP. Using GW6471, a selective PPAR- $\alpha$  antagonist, we investigated whether PPAR- $\alpha$  inhibition alters the effect of ATX. In a recent study, the absence of the PPAR- $\alpha$  gene significantly increased cerulein-induced pancreatitis and pancreatic injury (8). Furthermore, another recent study revealed that treatment with a PPAR- $\alpha$  agonist WY14643 ameliorated changes in lipase, amylase, and MPO activity and pathological scores in cerulein-induced AP model. The same study also demonstrated that these effects were completely abolished by co-administration of PPAR- $\alpha$  antagonist MK886. However, our results indicated that GW6471 did not abolish the positive impact of ATX biochemically or histologically. This result may be owing to ATX having a very strong antioxidant or protective effect or ATX not acting via PPAR- $\alpha$  pathway.

In conclusion, this study shows that inhibition of PPAR- $\alpha$  does not block the effect of ATX in cerulein-induced AP. ATX, a naturally occurring potent antioxidant, significantly reduces oxidative stress and protects from pancreatic damage. The antioxidant effect of ATX in the cerulein-induced AP model is not only related to its scavenging activity but also could be owing to its increasing effect on antioxidant defense enzyme expression. Thus, ATX can be used as a valuable therapeutic approach in AP.

**Ethics Committee Approval:** Ethics committee approval was received for this study from the Marmara University, Animal Care and Use Committee (Approval number: 76.2017.mar).

**Informed Consent:** N/A.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept – D.Ö., E.B.G.; Design – D.Ö., E.B.G.; Supervision – D.Ö.; Resource – D.Ö.; Materials – D.Ö., Ö.T., Ç.K., M.Y.; Data Collection and/or Processing – D.Ö., H.B., Ö.T.Ç.K., M.E.Ç., M.Y.; Analysis and/or Interpretation – D.Ö., E.B.G.; Literature Search – D.Ö., E.B.G.; Writing – D.Ö., E.B.G., H.B., Ö.T.Ç.K., M.E.Ç., M.Y.; Critical Reviews – D.Ö., E.B.G.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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