TIPS

The dipeptide carnosine alleviates acute pancreatitis in an experimental model

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Abstract

Acute pancreatitis (AP) is a severe inflammatory disorder with a significant risk of mortality. However, restricted pharmacological treatments are available against this complication. Carnosine is an endogenous dipeptide with various pharmacological effects, including antioxidative and anti-inflammatory properties. The current study was designed to evaluate the impact of carnosine in an experimental model of AP. For this purpose, mice received arginine (two 4 g/kg doses, one-hour intervals, i.p) to induce AP. Then, animals received carnosine (50, 100, and 200 mg/kg, i.p). Serum levels of amylase, lipase, and glucose were significantly increased (P<0.001) in the current AP model. Moreover, alterations in oxidative stress biomarkers in the pancreas, including ROS formation, decreased antioxidant capacity, lipid peroxidation, and glutathione depletion, were detected in the AP group (P<0.001). A significant increase in the pancreatic level of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) was also evident in the l-arginine-treated mice (P<0.001). The major pancreatic tissue histopathological changes in the current AP model were the infiltration of inflammatory cells to the pancreas tissue, fluid accumulation, and acinar cell vacuolization/necrosis (P<0.05). Carnosine significantly reduced serum biomarkers of pancreas injury, alleviated oxidative stress, decreased pro-inflammatory cytokine levels, and improved histopathological changes in the pancreas of mice with AP (P<0.001). These findings suggest that carnosine is a protective agent in pancreatitis, with its antioxidative and anti-inflammatory properties playing a pivotal role in its mechanisms of action. Further research is needed to confirm these protective effects in clinical studies and assess carnosine safety in AP.

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1. Introduction

Acute pancreatitis (AP) is a severe inflammatory disease that can lead to a high mortality rate of up to 50% (1-3). Symptoms of acute pancreatitis can vary from mild abdominal discomfort to a life-threatening condition caused by the release of pancreatic

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enzymes into the abdominal cavity (1, 2). Various factors such as gallstones, alcoholism, infections, certain medications, hypertriglyceridemia, and trauma have been implicated in the development of AP (1, 2, 4). AP could lead to serious complications such as systematic inflammatory conditions and multi-organ failure (5-7).

During acute pancreatitis (AP), several critical processes occur in pancreatic tissue. One of the most serious events is the activation of enzymes like amylase and lipase, which results in the autodigestion of the pancreas tissue (8). This autodigestion, induced by pancreatic enzymes, leads to severe accumulation of the inflammatory cells in the pancreas and organ damage (8). This severe inflammatory response could even extend to the surrounding tissues and organs (8). Moreover, AP can give rise to various other changes, including alterations in pancreas vasculature, the formation of fluid-filled sacs or abscesses, tissue necrosis, and organ failure (9, 10).

The management of AP primarily involves eliminating risk factors such as gallstone surgery, cessation of ethanol consumption, eradication of infectious diseases, and administering drugs such as anti-hyperlipidemic agents (10). Various other interventions have also been developed to address AP and its related complications. These interventions include intravenous fluid hydration, prevention of gut microbiota translocation and their products to the systemic circulation (e.g., by administration of probiotic bacteria such as Lactobacillus and Bifidobacterium strains, that may help maintain a balanced gut microbiota and enhancing gut barrier function), treatment of metabolic complications like hyperglycemia and hypocalcemia, and management of acute pancreatitis-associated sepsis through the use of antibiotics (10). Surgical interventions may also be necessary to remove pancreas tissue, such as parts of necrotized/damaged tissue. Pharmacotherapy for acute pancreatitis includes medications to alleviate pain, such as acetaminophen, tramadol, and meperidine, as well as to treat infections with drugs like ceftriaxone, ampicillin, and imipenem. However, there are currently no drugs available that directly target pancreas tissue or specifically treat acute pancreatitis.

A variety of experimental models for pancreatitis have been devised to study the disease's pathophysiology and potential treatments (11). Nonetheless, it has been discovered that certain models resolve rapidly and do not result in any fatalities (11). Models like choline-deficient-methionine-supplemented diet-induced pancreatitis also present challenges, including a high mortality rate and inconsistent outcomes (11).

Utilizing l-arginine to induce acute pancreatitis in mice is a recognized technique for studying the pathophysiology of the disease and exploring therapeutic interventions (11, 12). Studies have confirmed that l-arginine can trigger a predictable model of acute pancreatitis in C57BL6 mice (12). The involvement of oxidative stress in pancreas tissue injury is significant in the arginine-induced animal model (12-15). Additionally, there is a noticeable increase in inflammatory cells and immune response in the pancreas of mice with arginine-induced pancreatitis (13-15). These findings suggest that antioxidant compounds and anti-inflammatory agents have the potential to mitigate acute pancreatitis and its associated issues.

Carnosine is a naturally occurring dipeptide molecule composed of β-alanine and L-histidine. This peptide is found in high concentrations in skeletal muscle tissue, the brain, and other tissues throughout the body (16). Carnosine has garnered significant attention due to its biological functions and potential health benefits (16-18). Antioxidant properties, anti-glycation action, and anti-inflammatory activity of carnosine have been repeatedly highlighted in various experimental models (19-22). Interestingly, the protective effects of carnosine on the pancreas tissue, especially the beta cells of the pancreas, have been investigated (in diabetes experimental models) (23, 24). In these studies, it has been found that carnosine could significantly decrease oxidative stress and its complications in the pancreas tissue of diabetic models (23, 24). However, there is no data on the effect of carnosine on acute inflammation, tissue injury, and severe oxidative stress in acute pancreatitis that makes the current study novel.

As mentioned, oxidative stress and inflammation are basic mechanisms involved in the pathogenesis of AP (12-15). Meanwhile, carnosine is known for its anti-inflammatory and antioxidant properties in various experimental models (25, 26). Therefore, this study is designed to evaluate the effect of the dipeptide carnosine in an animal model of AP.

2. Materials and Methods

2.1. Chemicals

Carnosine was purchased from Sigma (St. Louis, MO, USA). 4,2 Hydroxyethyl,1-piperazineethanesulfonic acid, dimethyl sulfoxide (DMSO), D-mannitol, Bovine serum albumin (BSA), thiobarbituric acid, coomassie brilliant blue, sodium citrate, hydroxymethyl amino methane hydrochloride (Tris-HCl), and ethylenediaminetetraacetic acid were purchased from Merck (Darmstadt, Germany).

2.2. Animals

Male C57BL6/J mice (24±2 g) were obtained from the Experimental and Comparative Medicine Research Center of Shiraz University of Medical Sciences, Shiraz, Iran. The animals were housed in plastic cages containing wood-chip bedding, kept at an ambient temperature of 23±2 °C and a relative humidity of about 40%. The mice were given free access to tap water and were fed a standard rodent diet (Behparvar®, Tehran, Iran). Animals were handled according to the animal treatment protocol approved by Shiraz University of Medical Sciences ethics committee (Ethic code #IR.SUMS.AEC.1403.080). The ARRIVE guidelines for laboratory animal care and use were also followed (27).

2.3. Treatments

Animals were allotted to four groups (n=12/group). The treatments were as follows: A) Control (saline-treated; 2.5 ml/kg, i.p); B) l-arginine (two doses of 4 g/kg, i.p, 1 hour apart); C) l-arginine + Carnosine (50 mg/kg, i.p); D) l-arginine + Carnosine (100 mg/kg, i.p); and E) l-arginine + Carnosine (200 mg/

kg, i.p). L-arginine caused acute pancreatitis 72 hours after the last dose administration (12). Then, carnosine was administered daily to animals with AP for three consecutive days. Blood and tissue samples were collected on day four after the first dose of carnosine administration. The selection of the l-arginine-induced acute pancreatitis method was made per established protocols that had been previously established (12). The dose of carnosine was selected based on previous studies, including our research team's investigations on the pharmacological properties of this peptide in various experimental models (26, 28). It should also be mentioned that due to the small size of mice pancreas tissue, we had to use a larger number of animals per group to pool pancreatic tissues in some tests. Meanwhile, about n=4 animals/group were used for histopathological evaluations.

2.4. Plasma biochemistry

Blood samples were obtained from the abdominal aorta and placed in sodium citrate tubes. Subsequently, the samples were subjected to centrifugation at 3000 g for 20 minutes at 4 ºC to separate the plasma. The plasma was then analyzed for glucose, amylase, and lipase levels using an autoanalyzer device (Mindray BS-200®) and commercial kits (Pars-Azmoon®, Tehran, Iran).

2.5. Tissue histopathology and organ weight index

The pancreas organ weight index was derived using the formula: organ weight index = [wet organ weight (g)/body weight (g)] × 100. Following this calculation, the tissue samples were carefully preserved in a buffered formalin solution to ensure their proper conservation. Subsequently, sections of the pancreas tissues measuring 4 µm in thickness were prepared by embedding them in paraffin. These sections were then stained with hematoxylin and eosin (H&E) for further analysis. The histopathological lesions of the pancreas

tissue in the current study were scored based on previous investigations in this field (29).

2.6. Reactive oxygen species (ROS) formation

The levels of reactive oxygen species (ROS) in pancreas samples were determined by utilizing 2′,7′-dichlorofluorescein diacetate (DCF-DA) as a probe (30, 31). In brief, the pancreas tissue was homogenized in 2 mL of ice-cooled 40 mM Tris-HCl buffer (pH=7.4). Subsequently, 100 µL of the resulting tissue homogenate was combined with 1 mL of 40 mM Tris-HCl buffer and 5 µL of DCF-DA (with a final concentration of 10 µM). The samples were then incubated at 37ºC for 10 minutes in the dark. Finally, the fluorescence intensity was measured using a FLUOstar Omega® fluorimeter with an excitation wavelength of 485 nm and an emission wavelength of 525 nm (30, 32, 33).

2.7. Pancreas glutathione (GSH) content

Reduced glutathione (GSH) levels in the pancreas tissue homogenates were measured using the DTNB (5,5'-Dithiobis (2-nitrobenzoic acid)) method (30, 34). Briefly, 500 µL of tissue homogenate (10% w: v in 40 mM Tris-HCl buffer, pH = 7.4) was combined with 100 µL of TCA (50% w: v), thoroughly mixed, and then centrifuged (10000 g, 15 min, 4 °C). Subsequently, the supernatant was mixed with 1 mL of PBS (pH=8.9) and 800 µL of DTNB solution (0.1 M in PBS). The samples were well mixed and incubated at room temperature for 5 minutes (protected from light). Finally, the absorbance was assessed at λ=412 nm (30).

2.8. Lipid peroxidation in the pancreas

The levels of TBARS were measured to assess lipid peroxidation in the pancreatic tissue (30). The reaction mixture contained 500 µL of tissue homogenate (10% w: v in 40 mM Tris-HCl buffer, pH=7.4) and 1 mL of TBARS assay reagent (comprising thiobarbituric acid, phosphoric acid, and trichloroace-

tic acid at pH=2). After thorough mixing, the samples were heated at 100 °C for 45 minutes. Once cooled, the mixture was centrifuged at 10000 g for 10 minutes. The absorbance was then read at λ=532 nm using an EPOCH® plate reader from BioTek® Instruments in the USA (30, 35).

2.9. Ferric reducing antioxidant power (FRAP)

The FRAP assay measures the production of a blue-colored ferrous (Fe^{2+})-tripyridyltriazine complex from the colorless oxidized ferric (Fe^{3+}) form (30, 36). To prepare the FRAP reagent, a mixture of acetate buffer (25 mL of 300 mmol/L, pH=3.6), TPTZ (2.5 mL of 10 mM in 40 mM HCl), and ferric chloride (2.5 mL of 20 mM FeCl3) was created. Tissue homogenate samples (100 µL) were then combined with 900 µL of the FRAP reagent. After an incubation period (37 ºC, 5 minutes, in the dark), the absorbance at λ=593 nm was assessed using the EPOCH® plate reader (BioTek® Instruments, USA (30).

2.10. Pro-inflammatory cytokines in the pancreas tissue

The pro-inflammatory cytokine levels in the pancreatic tissue were measured using commercial kits (37), according to the instructions provided by Karmania-Pars-Gene™ (Kerman, Iran).

2.11. Statistical analysis

The normality of the obtained data was analyzed using the Kolmogorov-Smirnov test (the data has a normal distribution). Then, a one-way analysis of variance (ANOVA) with Tukey's multiple post hoc test at $P<0.05$ was utilized to compare various experimental groups. The pancreatic histopathological changes (non-parametric data) were evaluated using the Kruskal-Wallis test and the Mann-Whitney U test. A significance level of $P<0.05$ was considered statistically significant.
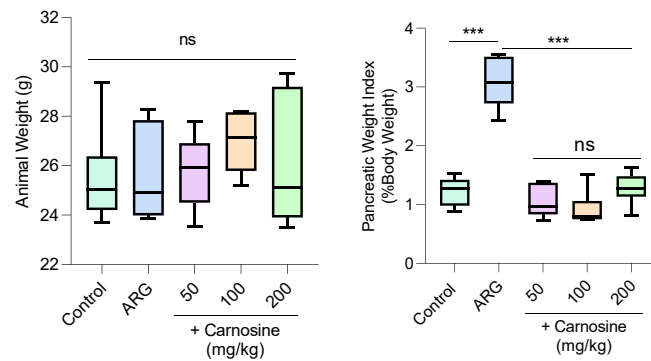
Figure 1. Animal weight and pancreatic weigh index in l-arginine (ARG)-induced acute pancreatitis. Data are represented as boxes and whiskers (min to max). Asterisks indicate a statistically significant difference (P<0.001). ns: not significant.

3. Results

Animal weight and pancreas weight index were monitored in the mice model of arginine-induced pancreatitis (Figure 1). Compared with the control group, no significant changes in the animals' weight were observed in the l-arginine-treated mice (Figure 1). However, there was a noticeable increase in the pancreas weight index in the arginine-treated mice compared to the control group (Figure 1). In the current pancreatitis model, it was discovered that carnosine (50, 250, and 500 mg/kg, i.p) significantly reduced the pancreatic weight index (Figure 1).

A significant elevation in serum amylase, lipase, and glucose levels in the current animal model of pancreatitis was demonstrated (Figure 2). The application of carnosine (50, 250, and 500 mg/kg, i.p) led to a signifi-

cant decrease in serum biomarkers of pancreas injury in arginine-treated mice (Figure 2). Notably, the influence of carnosine on serum biochemical measurements exhibited a dose-dependent relationship in this study (Figure 2).

Biomarkers indicating oxidative stress were assessed in the pancreas of mice treated with arginine (Figure 3). Notably, there was a marked rise in ROS levels and lipid peroxidation in the pancreas due to arginine-induced pancreatitis (Figure 3). Additionally, the levels of GSH and total antioxidant capacity in the pancreas of arginine-treated mice were significantly lower compared to the control group (Figure 3). The study revealed that carnosine (50, 250, and 500 mg/kg, i.p) effectively mitigated the oxidative stress biomarkers in the pancreas tissue of arginine-treated mice (Fig-
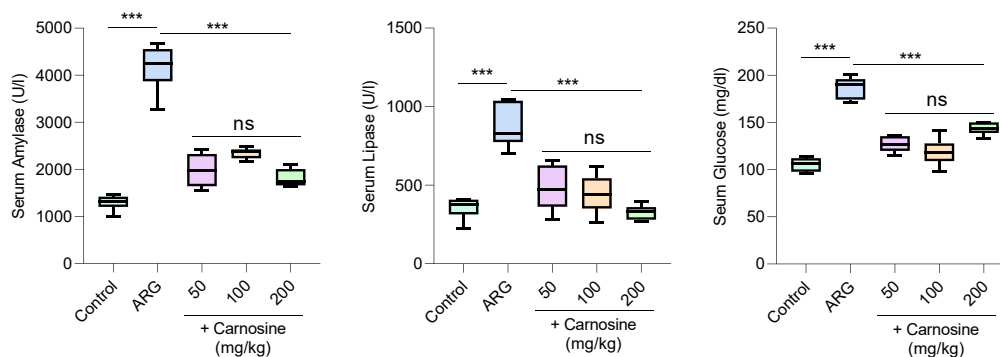

Figure 2. Serum biomarkers of pancreatic injury in l-arginine (ARG) model of acute pancreatitis. Data are represented as box and whiskers (min to max). Asterisks indicate a statistically significant difference (P<0.001). ns: not significant.
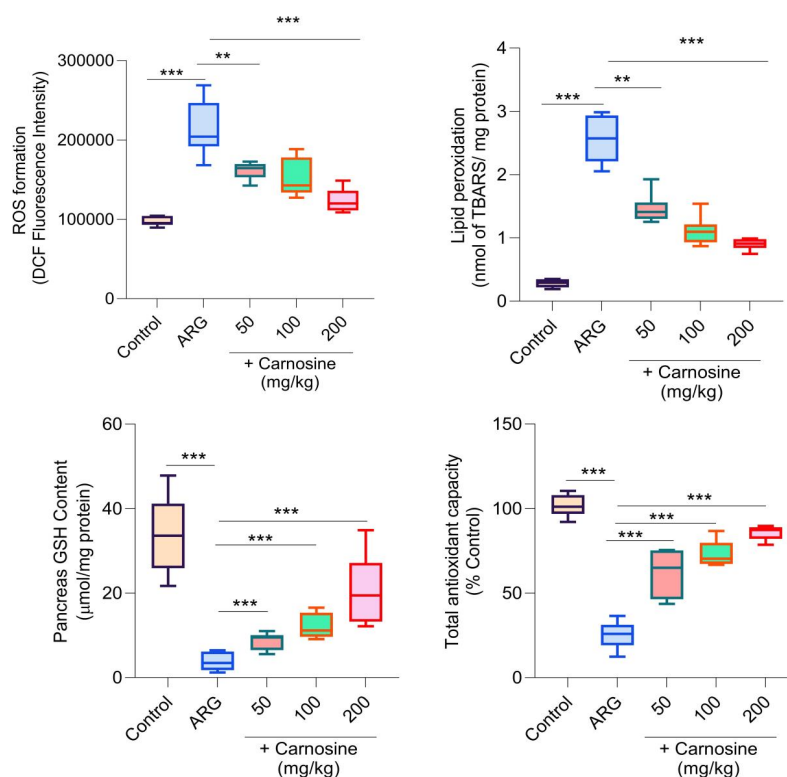
Figure 3. Biomarkers oxidative stress in the pancreas of l-arginine (ARG)-treated mice. ROS: Reactive oxygen species; DCF: 2′, 7′-Dichlorodihydrofluorescein; GSH: reduced glutathione. TBARS: Thiobarbituric acid reactive substances. Data are represented as box and whiskers (min to max). Asterisks indicate statistically significant differences (**P<0.01, ***P<0.001).

ure 3). It is important to note that the impact of carnosine on oxidative stress biomarkers in this particular animal model of pancreatitis showed a dose-dependent relationship (Figure 3).

Pancreatic concentrations of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were markedly elevated in arginine-induced pancreatitis when compared to the con-

trol group (Figure 4). The study revealed that (50, 250, and 500 mg/kg, i.p.) notably reduced TNF-α, IL-1β, and IL-6 pancreatic levels in mice treated with arginine (Figure 4). The impact of carnosine on pancreatic pro-inflammatory cytokine was dose-dependent in the current study (Figure 4).

In the current animal model of acute pancreatitis, the examination of pancreas tis-
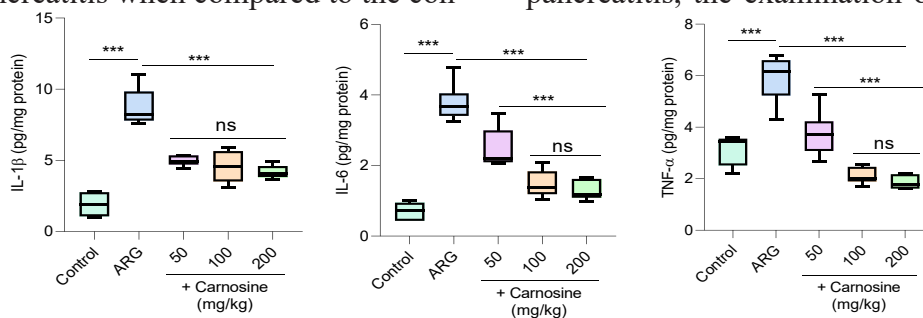

Figure 4. Pancreatic level of pro-inflammatory cytokines in the l-arginine (ARG)-induced pancreatitis animal model. Data are represented as box and whiskers (min to max). Asterisks indicate a statistically significant difference (P<0.001).

Table 1. Grades of pancreas tissue histopathological alterations in an animal model of acute pancreatitis.

Treatment	Acinar Cell Vacuolization	Acinar Cell Necrosis	Inflammation	Total Score
Control	0 (0, 0)	0 (0, 0)	0 (0, 0)	0
ARG	3 (3, 3) *	1 (0, 1) *	3 (2, 3) *	7
ARG + CARN 50 mg/kg	1 (1, 1) #	0 (0, 0) #	1 (1, 1) #	2
ARG + CARN 100 mg/kg	0 (0, 0) #	0 (0, 0) #	1 (1, 1) #	1
ARG + CARN 200 mg/kg	0 (0, 0) #	0 (0, 0) #	1 (0, 1) #	1

0 = absent; 1 = mild; 2 = moderate, and 3 = severe histopathological changes. The histopathological lesions of the pancreas tissue in the current study were scored based on previous investigations in this field (29). ARG: l-arginine; CARN: Carnosine. Data are represented as median and quartiles for six random pictures per group. * Indicates significantly different from the control group ($P < 0.05$). # Indicates significant difference as compared with arginine (ARG)-treated group ($P < 0.05$).

sue from arginine-treated animals showed notable acinar vacuolization, necrosis, and inflammation (Table 1). It was found that carnosine administration significantly decreased pancreas tissue histopathological changes in the current animal model of acute pancreatitis (Table 1).

4. Discussion

Acute pancreatitis (AP) is a severe clinical complication with a high mortality rate. Severe oxidative stress and inflammatory response play a crucial role in the pathogenesis of AP. Unfortunately, there is no specific pharmacological intervention against AP. The current study found that αKG supplementation significantly blunted pancreas tissue injury in an animal model of AP. The effects of αKG on oxidative stress and its associated complications, as well as its role in mitigating pancreas inflammation, seem to play an essential role in its protective properties.

It has been well-established that oxidative stress plays an essential role in the pathogenesis of acute pancreatitis (38-40). It has been found that oxidative stress could induce cellular injury, mitochondrial dysfunction, activation of inflammatory pathways, and impairment of antioxidant defense mechanisms in the pancreas (38). Oxidative stress induces lipid peroxidation, protein oxidation, and DNA damage, contributing to cellular injury and dysfunction in the pancreas tissue during AP (38). In this regard, several studies used antioxidants to protect pancreas tissue in AP (38). The antioxidant capacity of carnosine is a well-established feature of this peptide (16, 17, 19, 41-43). Carnosine has been shown to possess antioxidative properties through various mechanisms. The radical scavenging activity of carnosine has been mentioned in several studies (44, 45). It has been found that carnosine acts as a direct scavenger of free radicals, including superoxide anion ($O_2 \bullet -$), hydroxyl radical ($OH \bullet$), and peroxyl radical ($ROO \bullet$), as well as reactive nitrogen species (RNS) such as peroxynitrite ($ONOO-$) (16, 46). By its radical scavenging activity, carnosine neutralizes these free radicals, preventing them from injuring cellular components such as lipids, proteins, and DNA. It has been well-established that carnosine can inhibit lipid peroxidation, a chain reaction that occurs when ROS attacks unsaturated fatty acids in cell membranes, leading to membrane damage and dysfunction (47). By terminating lipid peroxidation chain reactions, carnosine helps preserve the structural integrity and fluidity of cell membranes (47). Carnosine also protects proteins from oxidative damage by scavenging ROS that targets protein side chains, such as reactive carbonyl groups, sulfhydryl groups, and aromatic amino acids (48, 49). The positive effects of carnosine on mitochondrial function, energy metabolism, and alleviation of mitochondria-originated ROS also have been well-established (50-53).

Carnosine can also modulate cellular

redox status by regulating the balance between oxidants and antioxidants within cells. Several studies mentioned that carnosine enhances the activity of endogenous antioxidant enzymes such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), which detoxify reactive species (54-56). Additionally, carnosine may influence the expression of antioxidant genes by activating nuclear factor erythroid 2-related factor 2 (Nrf2), a nuclear transcription factor that controls the antioxidant response (54-56).

The antioxidant properties of carnosine were evident in the current study by its effect in mitigating ROS formation and lipid peroxidation and enhancing cellular antioxidant defense in the pancreas tissue of the AP animal model (Figure 3). Hence, a key mechanism of carnosine protective effects in the current study is mediated by its effect on oxidative stress and related complications.

The anti-inflammatory property of carnosine is an interesting feature of this peptide (57, 58). It has been well-established that carnosine could help alleviate inflammation associated with conditions such as arthritis, asthma, and inflammatory bowel disease (59-62). Carnosine has anti-inflammatory effects, possibly modulating the immune response by hindering the production of pro-inflammatory cytokines and chemokines (59, 63). It has been well-characterized that carnosine modulates immune responses by controlling the activity of immune cells such as macrophages, neutrophils, and lymphocytes (64). Carnosine also inhibits the recruitment of inflammatory cells to sites of inflammation (64, 65). Mechanistically, it has been found that carnosine interferes with various signaling pathways involved in inflammation, such as the mitogen-activated protein kinase (MAPK) signaling and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (66). By inhibiting the activation of these signaling pathways, carnosine blocks the production of inflammatory mediators and reduces inflammation. Regu-lation of the activity of enzymes involved in inflammation is another interesting feature of carnosine. It has been found that carnosine regulates the activity of enzymes involved in inflammation, such as cyclooxygenase (COX) and lipoxygenase (LOX), which catalyze the production of inflammatory prostaglandins and leukotrienes (67). By inhibiting COX and LOX activity, carnosine reduces the synthesis of pro-inflammatory lipid mediators and attenuates inflammation. All these data mention the potent anti-inflammatory capacity of carnosine. In the current study, we found that carnosine provided significant anti-inflammatory properties in the pancreas tissue in AP model. This peptide significantly decreased pancreas level of proinflammatory cytokines (Figure 4). Moreover, the infiltration of inflammatory cells was significantly suppressed by carnosine in the pancreas tissue (Table 1).

5. Conclusion

In conclusion, the findings of this research indicate that carnosine can be considered a potent agent in combating AP. The impact of this naturally occurring peptide on the inflammatory response, oxidative stress, and related complications is crucial for its protective function in pancreatic tissue. Considering that carnosine is commonly used in humans at high doses (e.g., 1500 mg/day) without any significant adverse effects (68), it can be easily implemented in clinical settings. Therefore, further investigations are warranted to ascertain the efficacy of carnosine in clinical studies targeting AP.

Authors contributions

Funding source

Conflict of Interest

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