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Research Article

MODULATING EFFECT OF NARINGENIN, A NATURAL FLAVANONE OF CITRUS FRUITS ON THE ACTIVITY OF CASPASE-1 AND PROINFLAMMATORY CYTOKINES-A BIOCHEMICAL STUDY IN RATS ADMINISTERED ETHANOL AND CERULEIN

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ABSTRACT

Objective: Citrus fruits are commonly consumed for their health benefits and contain naringenin (Nrg) as the major flavanone. The objective was to investigate the anti-inflammatory effect of Nrg by applying ethanol (EtOH) and cerulein (Cer) induced model of pancreatitis.

Methods: Male albino Wistar rats were divided into four groups. Group 1 and 2 were fed normal diet. Group 3 and 4 were fed isocalorically adjusted diet containing EtOH (36% of total calories) for a total period of 5 weeks and also injected Cer intraperitoneally (20 µg/kg body weight) 3 times a week from 3rd week. In addition, Group 2 and 4 rats received daily 200 mg/kg body weight of Nrg orally from 3rd week till the experimental period of 5 weeks. Spectrophotometric assay of serum lipase, amylase, oxidative stress markers, and ELISA-based determination of inflammatory markers were carried out. The results were analyzed by one-way analysis of variance with *post-hoc* Bonferroni test.

Results: Nrg co-administration significantly decreased the levels of serum L/A ratio (18%, p=0.021), lipid peroxides, inflammatory markers interleukin-1β (IL–1β) (18%, p=0.044), IL-18 (58%, p=0.000) and caspase-1 (31.4%, p=0.000), improved antioxidant status and minimized histopathological alterations in EtOH-Cer administered rats.

Conclusion: The ability of Nrg to reduce caspase-1 activity and maturation of proinflammatory cytokines could be the underlying mechanism for its anti-inflammatory action in pancreatitis.

Keywords: Antioxidant, Anti-inflammatory, Interleukin-1β, Interleukin-18, Inflammation, Pancreatitis.

INTRODUCTION

Pancreatitis is a disease characterized by the inflammation of the pancreas which may be acute or chronic. Acute pancreatitis is a sudden inflammation of the pancreas in a short period. Chronic pancreatitis is a progressive destruction of pancreatic tissue leading to loss of both its endocrine and exocrine functions. The prevalence of chronic pancreatitis varies from 114 to 200 per 100,000 people in south India [1].

The major etiological factor associated with pancreatitis is chronic alcohol abuse while genetic mutations, smoking, high fat diet, tumors of the duct, abdominal trauma, viral infections, and several therapeutic drugs are the precipitating factors [2,3]. The pathophysiology of pancreatitis includes the activation of pancreatic enzymes within the acinar cells and their abnormal basolateral exocytosis leading to autodigestion of pancreas and also their release into the systemic circulation resulting in multiple organ failure [4].

The main symptoms of pancreatitis are abdominal pain, swollen abdomen, abdominal fullness, indigestion, steatorrhea, skin lesions, and mild jaundice [5]. The current treatment for pancreatitis includes control of pain by analgesics, improvement of maldigestion by intravenous fluids/nutrients, and management of other complications [6,7]. But these treatment strategies are limited and predominantly aimed at supportive therapy.

The pathology of pancreatitis is mainly due to the overproduction of proinflammatory cytokines [8]. The proinflammatory cytokines play a pivotal role in human inflammatory responses and so neutralizing these could inhibit inappropriate inflammation. The inflammasome, a multiprotein oligomer expressed in myeloid cells plays a significant role in the regulation of caspases involved in processing and secretion of cytokines [9].

A standard model to induce experimental pancreatitis is by administering ethanol (EtOH) and cerulein (Cer). Pancreas metabolises EtOH through oxidative and nonoxidative pathways. Oxidative EtOH metabolism results in the production of acetaldehyde and generation of reactive oxygen species (ROS) which in turn depletes the antioxidants. This results in oxidative stress and extensive tissue damage [10]. Cer, a cholecystokinin analog at supramaximally stimulating dose can induce pancreatitis very similar to human edematous pancreatitis in rodents. This is manifested by dysregulation of digestive enzyme production, cytoplasmic vacuolization, acinar cell death, edema, and infiltration of inflammatory cells into the pancreas [11,12]. Deng *et al.* in 2005 demonstrated that rats administered with 20 µg/kg body weight Cer developed recurrent pancreatitis characterized by hemorrhage, white blood cell infiltration, and fibrosis [13].

Flavonoids are polyphenolic compounds that are ubiquitous in nature and occur in fruits and vegetables. These flavonoids have shown antioxidant, antiatherogenic, and normolipidemic effects [14]. Naringenin (Nrg) is a naturally occurring trihydroxy flavanone present in grapefruits, tomatoes and other citrus fruits. It has been shown to possess potential anti-inflammatory, antioxidant and hypolipidemic properties both *in vivo* and *in vitro* [15]. Inflammation and oxidative stress have been implicated in the pathogenesis of many gastrointestinal diseases. Hence, the present study investigates whether Nrg could be an effective therapeutic remedy for pancreatitis by reducing the inflammation.

METHODS

Nrg was obtained from Sigma-Aldrich Chemicals Co. The ELISA kits for interleukin-1β (IL-1β) and caspase-1 were purchased from Abcam, and ELISA kit for IL-18 was purchased from Invitrogen. AxyPrep multisource total RNA miniprep kit was purchased from Axygen Biosciences, USA and cDNA reverse transcription kit was purchased from applied biosystems, USA. All other chemicals and solvents used for analysis were of analytical grade.

Animals and treatment

The work protocol was approved by the Animal Care Ethical Committee (CPCSEA) (XIII/VELS/PCOL/09/2000/CPCSEA/IAEC/08.08.2012). Adult male albino rats (Wistar) weighing 175-200 g were maintained on 12 hrs light-dark cycle at 22±2°C and 50% humidity. All animals were individually housed in polyacrylic cages and fed *ad libitum* standard rat chow obtained from M/S: Provimi Animal Nutrition India Pvt. Ltd., Bangalore, India, during the 1-week acclimatization period.

Rats were randomly assigned into four groups. Group 1 and 2 rats were fed *ad libitum* rat chow and water throughout the experimental period of 5 weeks. Group 3 and 4 rats were fed isocalorically adjusted diet containing EtOH (36% of total calories) for 5 weeks and subjected to intraperitoneal injection of Cer (20 µg/kg body weight) 3 times a week for the last 3 weeks [13]. A preliminary study was conducted by administering 10, 20, and 40 µg/kg body weight of Cer to EtOH diet fed rats and the dosage of 20 µg/kg body weight was selected based on the histopathological damage with relevant biochemical changes. Group 2 and 4 rats also received 200 mg/kg body weight of Nrg orally from 3rd week daily. At the end of the experimental period, all the animals were fasted overnight, anesthetized by intramuscular injection of ketamine hydrochloride (30 mg/kg body weight) and sacrificed by cervical decapitation. Blood was collected with/without anticoagulant and plasma/serum separated was stored for analysis.

Tissue homogenate preparation

Immediately after the animal sacrifice, pancreas was removed carefully, washed and homogenized in 0.1 M Tris HCl buffer (pH-7.4) and centrifuged at low speed to remove any cell debris. The supernatant was used for the determination of protein, lipid peroxides, and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).

Biochemical investigations

Determination of serum lipase activity

Lipase activity in serum was measured by the method of Lowry and Tinsley (1976) [16]. The lipolysis reaction was initiated with the addition of serum in 25 mL olive oil/triton X-100 emulsions as substrate. 0.3 mL subsamples of the reaction mixture were taken at predetermined time intervals and used for the assay of liberated free fatty acids spectrophotometrically at 715 nm. The activity of the enzyme was expressed as IU/L.

Determination of serum amylase activity

Amylase activity was determined by the method of Gomori [17]. The method was based on the activity of the enzyme on substrate (starch) and the measurement of maltose liberated by using Lugol's iodine solution. The color intensity was measured spectrophotometrically at 640 nm, and the enzyme activity was expressed as IU/L.

Estimation of lipid peroxides

The level of lipid peroxides in plasma and pancreas was determined by measuring thiobarbituric acid-reacting substances (TBARS) [18] and the value was expressed as nm/mL for plasma and nm/g protein for pancreas.

Estimation of antioxidant enzymes

GPx was assayed by the method of Flohé and Günzler [19]. The activity of GPx was expressed as nm of GSH oxidized/minute/mg protein. SOD activity was measured according to the method of Kakkar *et al.* [20]. The enzyme activity was expressed as units/mg protein. Decomposition of H_2O_2 in the presence of CAT was kinetically measured at 240 nm [21]. CAT activity was defined as the amount of enzyme required to decompose 1 µM of H_2O_2/minute. The enzyme activity was expressed as µM of H_2O_2 consumed/minute/mg protein.

Assay of caspase-1

Caspase-1 activity was determined spectrophotometrically using serum or pancreatic extract, prepared according to the method of Thornberry *et al.* [22]. Briefly, the pancreas was homogenized in a lysis buffer (25 mM HEPES [pH 7.5], 1 mM EDTA, 10 µg of aprotinin/mL, 10 µg of leupeptin/mL, 2 mM dithiotheritol) at 5 mL/100 mg of pancreas tissue. Extracts were centrifuged at 15,000 g for 30 minutes at 4°C, and the supernatant was centrifuged again at 200,000 g for 1 hrs at 4°C. The cytosol was used for caspase-1 activity measurements. The assay in undiluted serum or pancreas extract was performed as per the kit manufacturer's instruction.

Assay of IL-1β

The assay was performed according to manufacturer's instructions (ab100767). Standards or serum samples were pipetted into the wells precoated with IL-1β antibody and IL-1β present in the sample was bound to the well by the immobilized antibody. The wells were washed and biotinylated anti-rat IL-1β antibody was added. The unbound biotinylated antibody was washed and added horseradish peroxidase-conjugated streptavidin to the wells. The wells were washed again, and tetramethylbenzidine substrate solution was added to the wells. The intensity of the color developed was proportional to the amount of IL-1β present in the sample. The stop solution changes the color from blue to yellow and the intensity of the color was measured at 450 nm. The activity of IL-1β was expressed as pg/mL.

Assay of IL-18

The assay was carried out as per the instruction of kit manual (KRC2341). The serum sample and standards were pipetted into antibody immobilized wells and biotinylated secondary antibody was added after incubation. After removal of excess secondary antibody, streptavidin-peroxidase was added. The substrate solution was added to the bound enzyme to produce color. The intensity of this color was measured spectrophotometrically at 450 nm. The activity of IL-18 was expressed as pg/mL.

Estimation of protein

Protein concentration was determined in the tissue homogenate by the method of Bradford [23]. This was used to calculate the enzyme activity.

Histopathological analysis

For histopathology examination, the pancreatic tissues were excised and rinsed with ice-cold saline solution (0.9% sodium chloride) to remove blood and debris of adhering tissues. The tissues were then fixed in 10% formalin for 24 hrs. The fixative was removed by washing through running tap water and after dehydration through a graded series of alcohols; the tissue was cleaned in methyl benzoate and embedded in paraffin wax. Sections were cut into 5 µM thickness and stained with hematoxylin and eosin. After dehydration and cleaning, the sections were mounted and observed under light microscope.

Histology score was determined in each slide and the average score was calculated. Grading for edema was scaled as: 0 - Absent or rare; 1 - Edema in the interlobular space; 2 - Edema in the intralobular space; and 3 - The isolated island shape of pancreatic acinus. Inflammation was scored as: 0 - Absent; 1 - Mild; 2 - Moderate; and 3 - Severe. The acinar necrosis was as follows: 0 - Absent; 1 - Focal (<5%); 2 - And/or sub lobular (<20%); 3 - And/or lobular (>20%).

Statistical analysis

Data were analyzed using a commercially available statistics software package SPSS for window V.10.0 (SPSS Inc.). The statistical significance

of mean values between different groups was determined by applying one-way analysis of variance (ANOVA) with *post-hoc* Bonferroni test and the $p<0.05$ was considered as significant, and results were presented as mean±SD.

RESULTS

Analysis of serum and pancreatic tissue of experimental animals revealed the following results:

Effect of Nrg on serum lipase/amylase (L/A) ratio

Fig. 1 represents the ratio of serum amylase and lipase activities. The L/A ratio was found increased significantly (p=0.021) in EtOH-Cer treated rats, when compared to rats co-administered with Nrg (Group 4). Group 2 rats showed no significant change in comparison to control rats.

Effect of Nrg on TBARS level

Fig. 2 depicts the effect of Nrg on the level of TBARS in plasma (a) and pancreas (b). The EtOH-Cer treated rats (Group 3) showed a significant (p=0.000) increase in the TBARS level when compared to control the group. The level of TBARS was significantly decreased in Nrg co-administered rats than in EtOH-Cer control rats in both plasma (p=0.014) and pancreatic tissue (p=0.000).

Effect of Nrg on antioxidants level

Fig. 3 shows the activities of antioxidant enzymes in experimental animals. SOD (p=0.000), CAT (p=0.000) and GPx (p=0.001) activities were decreased significantly in EtOH-Cer treated rats when compared to normal control rats. Nrg co-administered rats in Group 4 showed a significant increase in the activities of SOD (p=0.002), CAT (p=0.022), and GPx (p=0.006) when compared to rats in EtOH-Cer treated Group 3.

Effect of Nrg on caspase-1

The effect of Nrg on the activity of caspase-1 is shown in Fig. 4. EtOH-Cer administration showed a significant increase in both serum and pancreatic caspase-1activity (p=0.000) when compared to normal rats in Group 1. In Nrg co-administered rats, the activity of caspase-1 decreased significantly in serum (p=0.000) and pancreas (p=0.008). Group 2 rats showed no significant change in caspase-1 activity in comparison to control rats.

Effect of Nrg on cytokines level

Fig. 5 depicts the level of serum cytokines IL-1β and IL-18 in experimental rats. The proinflammatory cytokines IL-1β (p=0.044) and IL-18 (p=0.000) were significantly decreased in the rats that received Nrg in addition to EtOH-Cer in comparison to rats that received EtOH-Cer alone.

Effect of Nrg on histology of the pancreas

Table 1 shows the histopathological score of pancreas in experimental rats. The pancreas of control rats showed normal architecture without inflammation and necrosis. EtOH-Cer administered rats showed interstitial edema, extensive inflammation with inflammatory cells, parenchymal cell necrosis, and hemorrhage. Nrg co-administration significantly (p=0.000) reduced the inflammatory changes, necrosis, and edema.

DISCUSSION

Chronic alcohol abuse has been implicated as the main predisposing factor for chronic pancreatitis in 70% of cases, while other factors such as smoking, obstructive disorders, genetic factors, and recurrent acute pancreatitis also play a significant role [2,24]. Cer acts as initiating factor for chronic pancreatitis while chronic alcohol abuse acts as a progression factor in worsening the disease. Hence, the combination of EtOH-Cer presents an equivalent to the pathology and manifestations of human pancreatitis [13].

Alcoholic pancreatitis is characterized by highly elevated level of lipase with moderate elevation in amylase activity and hence high L/A ratio. The measurement of serum lipase and amylase activity helps in diagnosing pancreatic cell injury. Abnormal activation and basolateral exocytosis of pancreatic enzymes occur in pancreatitis [4]. Hence, these enzymes are released from the pancreatic tissue into blood vessels

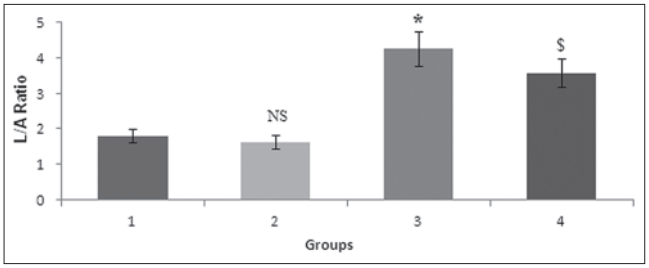

Fig. 1: The effects of naringenin on lipase/amylase ratio in the serum of control and experimental animals. Data were analyzed by one-way analysis of variance followed by *post-hoc* Bonferroni. Values are expressed as mean±standard deviation for six rats in each group. Statistical significance comparisons were made between Group 1 versus Group 2, Group 1 versus Group 3, and Group 3 versus Group 4. *p=0.000, $p=0.021, NS=Non-significant

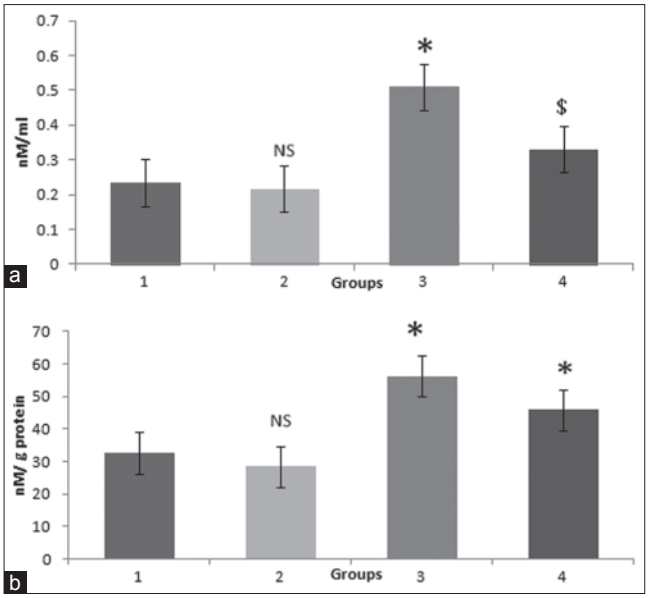

Fig. 2: The level of lipid peroxides in the plasma (a) and pancreas (b), respectively. Data were analyzed by one-way analysis of variance followed by *post-hoc* Bonferroni. Values are expressed as mean±standard deviation for six rats in each group. Statistical significance comparisons were made between Group 1 versus Group 2, Group 1 versus Group 3, and Group 3 versus Group 4. *p=0.000, $p=0.014, NS=Non-significant

Table 1: The effect of naringenin on histopathology scores in pancreas of experimental animals

Groups	Edema	Inflammatory cell filtrate	Acinar necrosis	Global score
1	0	0	0	0
2	0	0	0	0
3	2.9±0.3*	1.4±0.16*	1.79±0.15*	6.09±0.7*
4	0.5±0.06*	0.4±0.03*	0.25±0.02*	1.15±0.1*

Data were analyzed by one-way analysis of variance followed by *post-hoc* Bonferroni. Values are expressed as mean±SD for six rats in each group. Statistically significant variations are expressed as *p=0.000 for Group 1 versus Group 3 and Group 3 versus Group 4

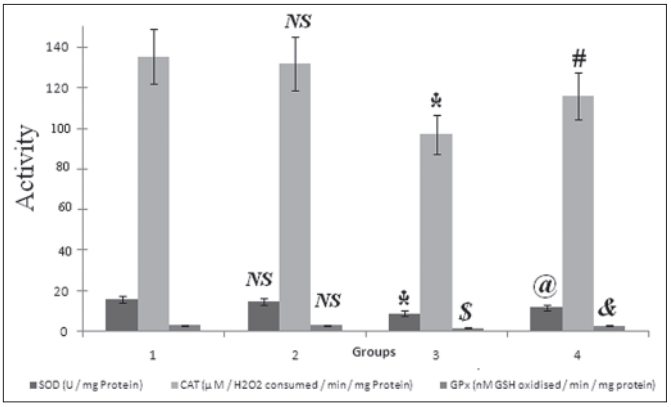

Fig. 3: The activities of antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase in control and experimental animals. Data were analyzed by one-way analysis of variance followed by *post-hoc* Bonferroni. Values are expressed as mean±standard deviation for six rats in each group. Statistical significance comparisons were made between Group 1 versus Group 2, Group 1 versus Group 3, and Group 3 versus Group 4. *p=0.000, $p=0.001, @p=0.002, #p=0.022, and p=0.006, NS=Non-significant

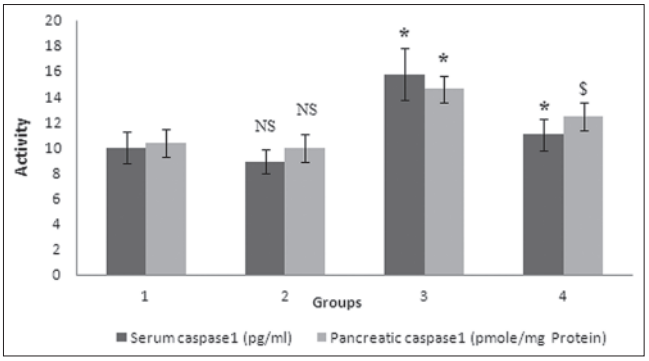

Fig. 4: The changes in the activity of caspase-1 in the serum and pancreas of control and experimental animals. Data were analyzed by one-way analysis of variance followed by *post-hoc* Bonferroni. Values are expressed as mean±standard deviation for six rats in each group. Statistical significance comparisons were made between Group 1 versus Group 2, Group 1 versus Group 3, and Group 3 versus Group 4. *p=0.000, $p=0.008, NS=Non-significant

instead of the pancreatic duct with a concomitant increase in the systemic circulation [25]. EtOH-Cer administration resulted in elevated L/A ratio indicative of the pancreatic injury and decrease in L/A ratio in Nrg co-administered rats show that Nrg was effective in reducing this alteration. This shows that Nrg protects the pancreas from injury caused by EtOH-Cer.

Lipid peroxidation is a crucial step in the pathogenesis of several diseases like pancreatitis that involve inflammation and tissue damage [26,27]. EtOH is extensively metabolized by the enzymes alcohol dehydrogenase, CAT and CYP2E1 to cytotoxic acetaldehyde which is further oxidized to acetate by aldehyde dehydrogenase or xanthine oxidase. These processes give rise to the formation of ROS and superoxide molecules and depletion of proteins that eliminate ROS. These factors increase the degree of lipid peroxidation during alcohol ingestion. The acetaldehyde and free radicals formed cause extensive tissue damage to the liver and pancreas [28]. The elevated level of TBARS in EtOH-Cer-treated animals showed the onset of lipid peroxidation during pancreatic cell injury. ROS formed by the metabolism of EtOH is known to aggravate the process of inflammation. The antioxidant nature of Nrg is well-proved in this study which

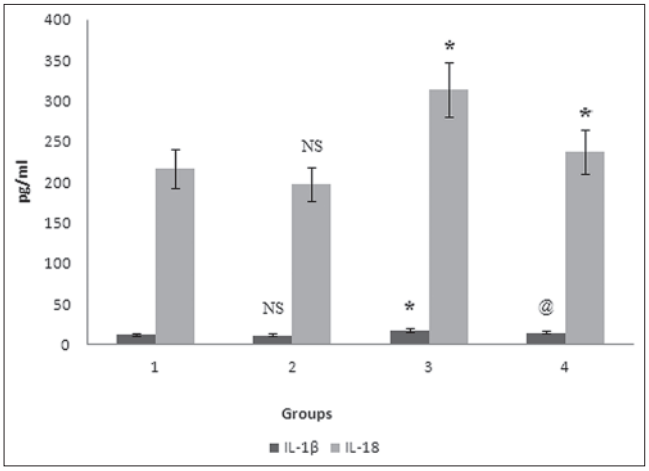

Fig. 5: The changes in the activity of interleukin-1β (IL-1β) and IL-18 in the serum of control and experimental animals. Data were analyzed by one-way analysis of variance followed by *post-hoc* Bonferroni. Values are expressed as mean±standard deviation for six rats in each group. Statistical significance comparisons were made between Group 1 versus Group 2, Group 1 versus Group 3, and Group 3 versus Group 4. *p=0.000, @p=0.044, NS=Non-significant

significantly reduced the level of TBARS. The concerted action of enzymatic antioxidants such as SOD, CAT, and GPx counteract the formation of toxic free radicals and protect the pancreas from their deleterious effects [29]. The present study also shows that these antioxidant enzymes that were depleted in the group 3 animals were significantly raised in animals co-administered with Nrg. Flavonoids like Nrg have been widely reported to have free radical scavenging activity and inhibitory effect on free radical generating enzymes [30]. The results show that Nrg plays an important role in protecting the pancreas by elevating the antioxidant enzymes and quenching the lipid peroxides and other free radicals formed.

The structure and functions of inflammasomes which sense endogenous danger signals [31] are the topics of current interest due to their pivotal role in the inflammatory cascade involved in innate immunity. Nod-like receptor pyrin domain 3 (NLRP3) is the inflammasome shown to be involved in the inflammatory responses during pancreatitis [32]. NLRP3 contains caspase recruitment domain (CARD) and pyrin domain (PYD) domains that associate with the ASC adaptor protein and render a protein platform for the activation of procaspase-1. Caspase-1 is a cysteine protease which effectively carries out the maturation and secretion of proinflammatory cytokines IL-1 β and IL-18 [33]. The increase in caspase-1 activity in EtOH-Cer treated rats supports the role of caspase-1 in inflammatory changes induced by EtOH-Cer. The anti-inflammatory nature of Nrg is demonstrated by the reduced activity of caspase-1 in Nrg and EtOH-Cer co-treated rats. Our study indicates that Nrg might have interacted with the CARD and PYD domains of inflammasome to regulate the activation of caspase-1.

Cytokines IL-1 β and IL-18 play a major role in the progression of inflammation. These cytokines are synthesized in an inactive form and are activated at the time of inflammation by caspase-1, which posttranslationally cleave the cytokine zymogens to their active forms. The matured IL-1 β and IL-18 are ready to involve in the process of inflammation as they are released into the interstitium. These cytokines also stimulate the synthesis and release of other proinflammatory mediators [34,35]. The results of the present study show that the cytokine levels were elevated significantly in rats treated with EtOH-Cer which indicates that inflammatory responses were triggered in these rats. The cytokine levels in Nrg co-administered rats were reduced significantly reiterating the anti-inflammatory nature of Nrg. Any anti-inflammatory drug should reduce the cytokine formation which is the first level prevention of inflammation in order to reduce tissue injury.

Nrg has also been reported to inhibit the production of proinflammatory cytokines [36,37] in adipocytes, microglia, and macrophages.

Nrg supplementation significantly reduced the level of edema, inflammation, and steatosis in pancreas. The anti-inflammatory effect of Nrg might be accounted for the histopathological score observed in pancreas.

CONCLUSION

Nrg shows potential as a good pancreato protective agent probably by reducing the formation of active cytokines and TBARS. The antioxidant property of Nrg and its ability to influence caspase-1 activity could be the underlying mechanism of its beneficial action against pancreatitis. However, further study in terms of its effect on inflammasome needs to be done.

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