Pre-Clinical Evolutionary Study of Alpha-Pinene in L-Arginine Induced Acute Pancreatitis in Rat.

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ABSTRACT

Objective: Screening of alpha-pinene on L-arginine induced acute pancreatitis model. **Material and Methods:** In this model the acute pancreatitis was determined at 24h by determination of pancreatic wet weight/body weight ratio, nitrate/nitrite levels, lipid peroxidation (thiobarbituric acid reactive substances (TBARS)], proinflammatory cytokines [tumor necrosis factor (TNF)-α, C-reactive proteins (CRP) and interleukin (IL)], pancreatic myeloperoxidase (MPO) activity and serum levels of amylase and lipase. **Results:** Administration of L-arginine had induced pancreatitis and this was characterized by pulmonary complication including altered pancreatic wet weight/body weight ratio, nitrate/nitrite levels, proinflammatory cytokines, TBARS, MPO and serum levels of amylase and lipase. Treatment with alpha-pinene (100 and 200mg/kg) dose significantly attenuated the L-arginine-induced pancreatitis and related complication. **Conclusion:** The histological findings proved the amelioration of pancreatic injury by alpha-pinene and biochemical parameters proved anti-inflammatory and antioxidant property of alpha-pinene.

Keywords: Acute pancreatitis, Anti-inflammatory, Cytokines, L-arginine, alpha-pinene.

INTRODUCTION

Pancreatitis is defined as inflammation of pancreatic gland. Although pathogenesis is not yet clear, though oxidative stress, microcirculatory disturbances and leukocyte activation are major constituents of acute pancreatitis. This further activates digestive proteases, leukocytes and inflammatory cell infiltration. This leads to release of various kinds of inflammatory mediators, nitrogen species and reactive oxygen species. Several factors are responsible for the AP, like alcohol, gallstones, hereditary pancreatitis, hypercalcemia, hyperlipidemia, malnutrition, abdominal trauma, penetrating ulcers, malignancy, drugs like steroids, sulfonamides, furosemide, thiazides, infections like mumps, coxsackie virus, mycoplasma pneumoniae, ascaris, Clonorchis, and structural abnormalities like choledochocele and pancreas divisum. Repeated attacks of acute pancreatitis have the potential to develop into chronic pancreatitis or pancreatic cancer characterized by fibrosis and loss of acinar cell function.[1,2] Despite medical treatment, the lethality of severe acute pancreatitis is still high (20–30%). Mizunuma, 1984 had developed experimental models of necrotizing pancreatitis by intraperitoneal administration of a high dose of L-arginine in rats. This model produces dose-dependent acinar cell necrosis and is highly reproducible, hence used in preclinical studies. Use of semi-synthetic and synthetic treatment may leads to addiction and photosensitivity skin reactions. These semi-synthetic and synthetic treatments are very expensive and not reliable. Hence, there is need to explore potential antioxidant and anti-inflammatory agents available from natural sources, which are cost-effective and have several advantages than the synthetic and semisynthetic compounds. Antioxidant, anti-inflammatory

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and anti-carcinogenic activities of alpha-pinene have been reported.[3,4] Alpha-pinene is one of the common monoterpenoids emitted from several aromatic plants and has growth-inhibitory activity and inhibit the nuclear translocation of NF-kB activity by upregulation of IkBα expression.[5] Hence in this study the non-toxic dose of alpha-pinene (100 and 200mg/kg) have been used to evaluate the potential effect to ameliorate pancreatic injury induced by L-arginine.

MATERIALS AND METHODS

Animals

Male Wistar rats (30) weighing, 180–200g obtained from Mahaveer Enterprises, Hyderabad was maintained at a constant room temperature (23±2°C) with 12:12h light-dark cycles and free access to water and standard laboratory chow. The rats were randomly divided into 5 groups of 6 in each and experiments were performed after 12h of fasting. All the experimental procedures were carried out in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The study was reviewed and approved by the Institutional Animal Ethics Committee (320/CPCSEA dated 03-01-2001), G. Pulla Reddy College of Pharmacy, Hyderabad, India.

Chemicals: L-arginine (Sigma Aldrich Co Pvt Ltd, USA), alpha-pinene (DestilacionesBordasChinchurreta SA), hexadecyltrimethylammonium bromide (HETAB) (Sigma Aldrich Co Pvt Ltd, Switzerland), o-dianisidinedihydrochloride, thiobarbituric acid (TBA) (Sigma Aldrich Co Pvt Ltd, Germany), Griess reagent (Sigma Aldrich Co Pvt Ltd, Germany) and vanadium trichloride (Sigma Aldrich Co Pvt Ltd, USA) were procured from Sigma Aldrich Chemical Co. All other chemicals and reagents were of highest commercial grade available locally.

L-arginine powder: prepared as a solution by dissolving in 0.9% saline to a final concentration of 500mg/mL and the pH was adjusted to 7 with 5N HCl. Alpha-pinene was prepared as a solution by dissolving in 3% tween 80 and 0.9% saline to a final concentration of 100 and 200mg/mL and the pH was adjusted to 7 with 0.1N NaOH.

L-arginine-induced pancreatitis model: Acute pancreatitis was induced in five groups of rats by two intraperitoneal (ip) injections of L-arginine (2.5g/kg, 1h apart). One hour following the last injection of L-arginine, the rats were treated orally as follows: Group: 1 received the vehicle (3% Tween 80) of alpha-pinene (vehicle control); Group: 2 and 3 were treated with alpha-pinene (100 and 200mg/kg, respectively). Group: 4 acted as positive control and received methylprednisolone (30mg/kg), all in a volume of 10mL/kg and Group: 5, received saline (0.9%, NaCl, ip) in place of L-arginine and served as a normal control. After 24h of the last injection of L-arginine or saline, a midline laparotomy was performedin rats under ether anesthesia and blood samples were collected from the inferior vena cava, the rats were then exsanguinated, the whole pancreas was quickly removed and stored at −70°C until use. The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema (mg/g).

MACROSCOPIC EVALUATION

Pancreas weight/body weight ratio: The pancreas was removed immediately after the blood collection, trimmed free of fat and weighed. The pancreatic weight/body weight ratio (mg/g) was calculated for each animal, to estimate the level of pancreatic edema.[6]

Serum analysis: For serum analysis, blood samples were centrifuged at 3000g at 4°C for 10min. The serum amylase and lipase were determined by the enzyme-colorimetric method using Automated Hitachi Analyzer, with the use of commercial kits for amylase (Rapid diagnostics), lipase (Accurex diagnostics), C-reactive protein and interleukin-α and expressed as U/dl.[7]

Biochemical estimations: Pancreatic total protein content[6] was determined. Myeloperoxidase activity,[8] nitrate/nitrite level,[9,10] TBARS level,[11] catalase activity,[12,13] SOD level[14] and reduced GSH level[15] were measured.

Histopathological evaluation: Pancreas was removed immediately and part of it was fixed in 10% neutral buffered formalin and embedded in paraffin by standard methods. Paraffin sections of 5µm thicknesses were cut and stained with haematoxylin and eosin, assessed under dark field microscope and examined blind by a morphologist for grading histopathological changes. Pancreatic damage was assessed and scored by grading acinar cell degeneration, interstitial inflammation, edema, and haemorrhage as described by schmidt's standards[16,17] with modification as follows: Grading for edema was scaled as 0: absent or rare; 1: edema in the interlobular space; 2: edema in the intralobular space; 3: isolated island shape of pancreatic acinus. Inflammation was scored as 0: absent; 1: mild; 2: moderate; 3: severe. Acinar cell necrosis was scored as 0: absent; 1: mild; 2: moderate; 3: severe. Parenchyma haemorrhage was scored as 0: absent; 1: mild; 2: moderate; 3: severe. The maximum score for acinar cell damage was 12.

Statistical analysis: Statistical analysis was performed by one way ANOVA followed by Newman Keuls as post-hoc test using Graph pad Prism 5. Values were presented as mean±SE. The difference was considered to be statistically significant when $P<0.05$.

RESULTS

Serum biochemical parameters and pancreatic edema: Induction of pancreatitis resulted in significant raise in the serum amylase, lipase and pancreatic edema. Treatment with alpha-pinene (100 and 200mg/kg) dose dependently decreased the serum amylase, lipase and pancreatic edema (Table 1).

Pancreatic MPO and total protein—Induction of pancreatitis resulted in significantly increased the pancreatic MPO and decreased the pancreatic total protein levels. Treatment with alpha-pinene (100 and 200mg/kg) dose dependently reversed the change in pancreatic MPO and total protein levels (Table 1).

Pancreatic, lung, liver and kidney MDA, nitrate/nitrite, GSH and antioxidant enzymes catalase and SOD: Induction of pancreatitis resulted in a significant raise in MDA, nitrate/nitrite, catalase and SOD and decline in GSH levels. Treatment with alpha-pinene (100 and 200mg/kg) dose dependently reversed the change in MDA, nitrate/nitrite, catalase, SOD and GSH levels (Table 1).

Assessment of interleukins and C- reactive protein: Induction of pancreatitis resulted in a significant raise in interleukins, TNF-α and C- reactive protein. Treatment with alpha-pinene (100 and 200mg/kg) dose dependently decreased the interleukins and C- reactive protein (Table 2).

Pancreatic histology: Histological examination of normal control group (saline treated) showed normal architecture and absence of edema, neutrophil infiltration, hemorrhage and necrosis (Fig 1). Whereas, pancreatic

Table: 1 Effect of Alpha-pinene on Pancreas Weight, Total Body Weight, Serum Amylase, Serum Lipase, Total Nitrate, Total Protein, MDA, MPO and SOD After L-arginine Induced Acute Pancreatitis

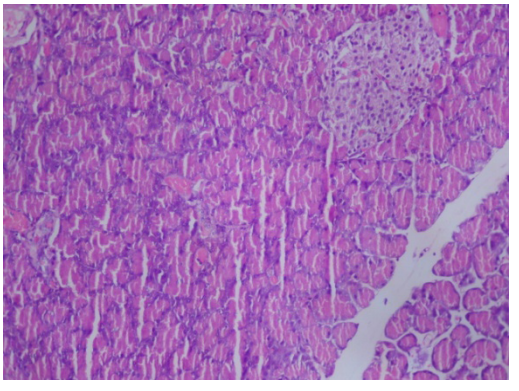
Parameter/Groups	N.C	D.C	STD	PIN 100	PIN 200
Pancreas weight	870.3±15.36	1015±19.0*	911.3±18.83α	862±83.67α	869±80.17α
Total body wt	187.5±4.889	192±3.578*	191.2±3.60	188±18.85	174±18.7
Pancreatic ind (x10−3)	4.652±1.26	5.286±1.57	4.769±1.32	4.58±1.28	4.99±1.24
Serum Amylase	2000±85.63	7667±349*	3317±110.8α	2933±527.9α	3200±46α
Serum Lipase	191.7±4.014	566.7±30.84*	346.7±39.21α	233.3±27.33α	250±24.49α
Total Nitrate	11.87±1.372	16.07±1.462*	7.06±0.39α	0.85±0.796α	0.71±0.853α
Total Protein	0.73±0.032	0.355±0.037*	0.91±0.067α	0.73±0.02α	0.82±0.05α
Kidney GSH	0.47±0.011	0.284±0.04*	0.71±0.014α	0.10±0.049	0.10±0.061α
Liver GSH	0.48±0.010	0.284±0.04*	0.67±0.03α	0.10±0.049	0.08±0.060γ
Lung GSH	0.50±0.020	0.284±0.04*	0.67±0.03α	0.08±0.039γ	0.10±0.047β
Pancreas GSH	0.48±0.011	0.284±0.04*	0.67±0.03α	0.10±0.049	0.08±0.060α
Kidney MDA	105.6±13.67	170.9±10.45*	135.2±16.42β	121.6±17.5α	129.4±13.55α
Liver MDA	105.6±13.67	170.9±10.45*	135.2±16.42γ	119.6±17.54α	126.9±16.51β
Lung MDA	14.95±0.66	17.09±1.04	9.82±1.04β	13.23±1.291α	14.24±1.62α
Pancreases MDA	1.62±0.6615	1.04±0.4267*	2.39±0.9756α	1.75±0.71α	1.35±0.55β
Pancreas MPO	4.75±2.1	32.8±4.7*	6.2±2.2α	19.2±3.7α	14.2±3.7α
Lung MPO	7.7±0.9402	38.92±2.259*	10.62±1.512α	26.15±3.48α	16.98±2.043α
Liver MPO	7.883±0.5419	31.73±2.223*	11.6±1.913α	28.2±3.426α	17.38±1.533α
Kidney MPO	7.883±0.5419	32.52±4.093*	6.953±1.043α	21.65±2.294α	15.1±2.494α
Pancreas Catalase	0.33±0.13	0.41±0.1707*	0.19±0.07	0.18±0.07α	0.12±0.05α
kidney SOD	2.33±0.95	1.50±0.6146	2.61±1.06 βα	1.63±0.66	1.171±0.47
Liver SOD	0.83±0.34	1.01±0.4153	1.97±0.80α	1.323±0.54β	1.63±0.66
Lung SOD	1.04±0.42	1.03±0.42*	3.44±1.40α	1.04±0.42	2.25±0.91α
Pancreases SOD	2.09±0.85	1.51±0.6191*	1.49±0.61α	1.50±0.614γ	1.04±0.42

*p < 0.0001 when compared with normal control, αp< 0.0001, βp< 0.001, γp< 0.01 when compared with disease control group. PI100: alpha-pinene 100 and PI200: alpha-pinene 200mg/kg. [Values are mean ± SEM from 6 animals in each group]

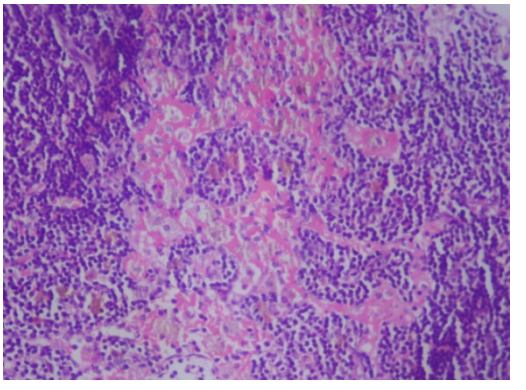
Table: 2 Effect of Alpha-pinene on IL-6, TNF-α and CRP After L-arginine Induced Acute Pancreatitis

Parameter/Groups	NC	DC	PIN1	PIN2
IL-6	29.3±1.569	90.48±1.689*	66.9±1.131	35.35±2.464γ
TNF-a	19.33±1.541	26.32±3.036	21.9±1.987	17.35±1.383
CRP	415.3±7.762	16403±119*	7436±63	562.8±17.44γ

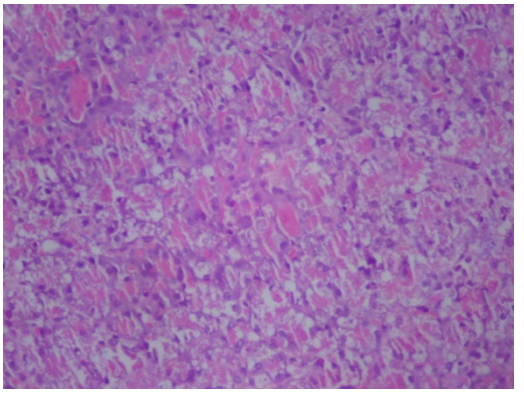
N.C- Normal Control, D.C- Disease Control, PI100: alpha-pinene 100 and PI200: alpha-pinene 200mg/kg. *P < 0.0001 when compared with normal control, γP< 0.01 when compared with disease control group.



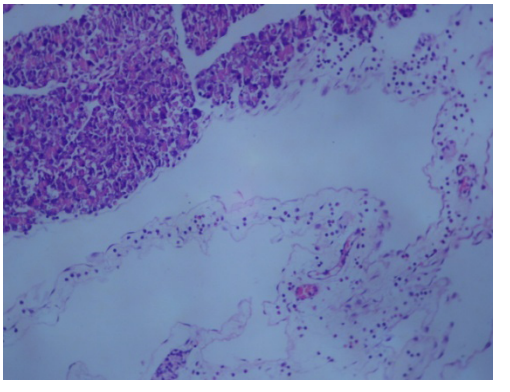
A. Normal Control



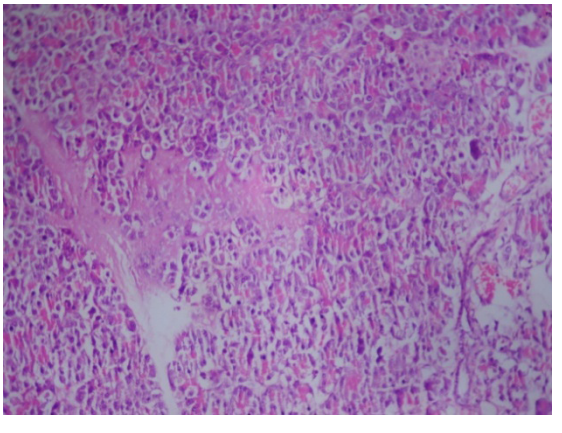
C. Disease control 24 hrs haemorrhage



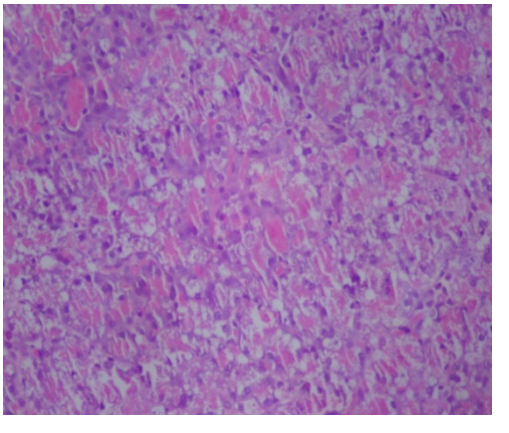
B. Standard Control



D. Disease control 24 hrs Oedema & necrosis



E. Alpha-pinene 100mg/kg, ip, 24 hrs



F. Alpha-pinene 200mg/kg, ip, 24 hrs

sections of disease control group showed extensive tissue damage characterized by acinar cell degeneration, necrosis, edema, mononuclear cell infiltration, hemorrhage and thus received significantly higher scores. Treatment with alpha-pinene (100 and 200mg/kg) and methyl prednisolone (30mg/kg) ameliorated the inflammation, edema and more significantly acinar cell degeneration and necrosis and protected the pancreas from L-arginine induced damage. Treatment with alpha-pinene dose dependently decreased the total pathological scores compared to disease control group.

Histopathology: Fig. 1-Effect of alpha-pinene on pancreatic histopathological changes after L-arginine induced acute pancreatitis [(A) normal control, (B) standard control, (C) disease control 24h hemorrhage, (D) disease control 24 h edema and necrosis, (E) alpha-pinene 100mg/kg, ip, 24, (F) alpha-pinene 200mg/kg, ip, 24h] (H&E×200)

DISCUSSION

In the present study alpha-pinene (200mg/kg) significantly attenuated pancreatitis in L-arginine induced acute pancreatitis model. In earlier report of Robbins,[2] it was stated that, the serum amylase and lipase are increased in the disease state. In consistent with previous reports alpha-pinene has significantly reduced the raised level of acinar cell necrosis, serum amylase and lipase.[5,18,19] One of the important diagnostic markers for acute pancreatitis are serum amylase and lipase, which rise within four to eight hours of initial attack and will be peak at 24 hours. Treatment with alpha-pinene decreased the serum amylase and lipase levels, indicating protective effect of alpha-pinene at early stage of the disease progression. Pancreatic markers like MPO, MDA, nitrite, catalase and SOD, increases at the time of acute pancreatitis. With consistent with previous, alpha-pinene decreased the level of MPO, MDA, nitrite, catalase, SOD and marker of neutrophil infiltration.[20,21] In agreement with previous reports[6,22] induction of pancreatitis with L-Arginine increased the pancreatic MPO levels. Inhibition of the neutrophil infiltration can attenuate the pancreatic injury.[23] Treatment with alpha-pinene significantly decreased the pancreatic MPO levels probably due to its anti-inflammatory action. One of the lipid peroxidation marker, which is elevated in L-arginine treated rats. Lipid peroxidation is a process mediated by free radicals, which results in impairment of the membrane functional and structural integrity[7,24,25] resulting in oxidative deterioration of polyunsaturated fatty acids of cell membrane. It could be attributed to the accumulation of free radicals proposed to be generated by L-arginine. The change in levels of catalase and SOD remains controversial. Czako and Takacs[25] reported the fall in these enzyme levels at 24 h. However, Szabolcs[24] reported the raised levels of these enzymes. In consistent with Robbins,[2] in the present study significant increase in SOD and catalase level was observed. It indicates that oxidative stress caused by L-arginine may up-regulate the activity of antioxidant enzymes to facilitate rapid removal of accumulated reactive oxygen and nitrogen species.[24] It is well known that GSH is found to be decreased in L-arginine treated rats indicating enhanced oxidative stress as the disease progresses.[26] The role of NO in the initiation and progression of acute pancreatitis remains controversial. Some studies[27–30] reported that NO increase the pancreatic blood flow and/or secretion in response to endothelium derived NO and ameliorates the pancreatic dysfunction, others suggested that NO aggravates pancreatic oxidative stress and damage. In agreement with previous reports,[30] in the present study significant increase in NO and pancreatic edema was observed in L-arginine received rats. Takacs[31] demonstrated that, administration of excess L-arginine could induce iNOS activity and increase the NO levels in pancreas. The raised levels of NO can increase vascular/micro capillary permeability and may contribute to the pancreatic edema and acinar cell damage. Alpha-pinene has significantly restored the pancreatic MDA, nitrite, edema, catalase, SOD and GSH in L-arginine received rats. Passaglia[32] stated that acinar cells are the protein factory of the body. In acute pancreatitis, catabolism of proteins could increase up to 80%. Consequently, a sharp decline in protein content was observed in pancreas. In consistent with report from Sidhu,[7] pancreatic total protein content, a marker of the tissue damage was found to decrease in L-arginine received rats. Treatment with alpha-pinene significantly increased the total protein content. It is well known that the extent of pancreatic tissue damage in acute pancreatitis correlates with the levels of inflammatory mediators and free radicals. In agreement with previous reports[7,33] in the present study, histopathological assessments revealed that, induction of pancreatitis resulted in pancreatic damage characterized by acinar cell necrosis, mono nuclear cell infiltration, edema and haemorrhage. Treatment with alpha-pinene protected the pancreas from L-arginine induced injury. In conclusion, the present study suggests that treatment with alpha-pinene significantly ameliorated the severity of L-arginine induced pancreatitis by reducing the neutrophil infiltration and oxidative stress markers and this effect may be due to antioxidant and anti-inflammatory properties of the alpha-pinene.

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