Ameliorating Effect of Eugenol on L-Arginine Induced Acute Pancreatitis and Associated Pulmonary Complications in Rats

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Abstract: Oxidative stress is one of the major causative factors for development of acute pancreatitis and associated pulmonary complications. There is no specific therapy for acute pancreatitis, so there is need to develop novel and safe therapeutic agents. With this background we hypothesized that treatment with eugenol, a potential antioxidant, may show beneficial effect on acute pancreatitis. Aim of the present study was to evaluate the protective effect of eugenol on L-arginine induced acute pancreatitis in rats. Wistar rats were divided into 4 groups (n = 12), group 1: received saline, group 2: received L-arginine (2.5 g kg⁻¹, i.p. two times with one hour interval)+Vehicle, group 3 and 4: received L-arginine+eugenol 100 and 200 mg/kg/day, p.o. respectively. Treatment was started one hour after the last injection of L-arginine and continued till the day of sacrifice. All the animals received their respective drugs two h before the sacrifice. In all groups, half of the animals were sacrificed at 24 h and remaining half were sacrificed at 72 h after the last injection of L-Arginine for estimation of biochemical parameters (serum amylase, serum lipase; pancreatic edema, malondialdehyde, superoxide dismutase, catalase and reduced glutathione; lung malondialdehyde) and histological studies of pancreas and lungs. Administration of L-Arginine induced the pancreatitis and associated pulmonary complications characterized by elevated levels of serum amylase and lipase, pancreatic edema, total protein, malondialdehyde, superoxide dismutase, catalase and reduced glutathione; lung malondialdehyde; histocorarchitectural changes in pancreas and lungs. Treatment with eugenol (100 and 200 mg kg⁻¹) dose dependently ameliorated the altered biochemical parameters and histoarchitectural changes in both pancreas and lungs at both 24 and 72 h intervals. In conclusion, the present study suggests that treatment with eugenol significantly ameliorated the L-arginine induced pancreatitis and associated pulmonary complications probably due to its antioxidant property.

Key words: Acute pancreatitis, L-arginine, eugenol, oxidative stress

INTRODUCTION

Acute pancreatitis is sudden inflammation of the exocrine pancreas with variable severity ranging from mild self-limiting condition to severe form; the later is associated with mortality particularly in the presence of systemic and pulmonary complications (Brown and Pitchumoni, 2006). Although its pathophysiology is not fully understood, oxidative stress has been reported to play a major role in determining the severity of pancreatitis (Szabolcs et al., 2006; Frossard, 2003). Despite extensive research, there are no specific therapies for acute pancreatitis. Several studies and meta-analysis evaluated the efficacy of somatostatin, gabexate mesylate and octreotide suggest a slight trend towards benefits (Andriulli et al., 1998; Karakoyunlar et al., 1999; Paran et al., 2000). However the present treatment is mainly aimed at supportive and symptomatic relief with the aid of analgesics, anti-inflammatory agents and steroids. As a result of the limitations of conventional therapy, there is need to develop novel and safe therapeutic agents to treat acute pancreatitis.

L-arginine induced pancreatitis is an experimental model of severe necrotising acute pancreatitis. L-arginine induces pancreatitis and significant associated lung injury mainly through oxygen and nitrogen derived free radical generation (Czako et al., 1998; Varga et al., 1997; Hardman et al., 2005). This model is highly reproducible, non-invasive and produces dose dependent acinar necrosis. Biochemical parameters & histological changes in pancreas are similar to the human disease, therefore ideal for studying protective effect of different drugs on...
acute pancreatitis (Hegyi et al., 2004). In view of these valuable observations, various studies reported that treatment with α,β-amyrin (Melo et al., 2010), pentoxifylline and alpha lipoic acid (Abdin et al., 2010), melatonin (Szaboles et al., 2006; Sidhu et al., 2010), hydrogen rich saline (Chen et al., 2010) and selenium (Harchman et al., 2005) ameliorates the L-arginine induced acute pancreatitis in rats by virtue of their antioxidant and/or anti-inflammatory properties.

Eugenol (2-allyl-4-methoxy phenol), a naturally occurring phenolic compound, is a major component of clove oil, nutmeg seed and exists to a lesser extent in oils of several other plants. Previous studies reported that eugenol possess antiinociceptive (Danieil et al., 2009), gastro protective (Morsy and Fouad, 2008), hepato protective (Nagababu et al., 1995), nephro protective (Sa'd, 2011) and anticancer (Kaur et al., 2010) properties by virtue of its antioxidant property. However, no study has reported the protective effect of eugenol on L-arginine induced pancreatitis. Aim of the present study was to evaluate the potential of eugenol to ameliorate the pancreatic injury induced by L-arginine in rats.

MATERIALS AND METHODS

Drugs and chemicals: L-arginine, Hexadecyltrimethylammonium bromide (HETAB), o-dianisidine dihydrochloride, Thiobarbituric acid (TBA), griess reagent and vanadium trichloride were purchased from sigma-aldrich chemical co. Eugenol was procured from HIMEDIA, Mumbai and all other chemicals and reagents were the highest commercial grade available.

L-Arginine powder: Prepared as a solution by dissolving in 0.9% saline to a final concentration of 500 mg mL⁻¹ and the pH was adjusted to 7 with 5 N HCl.

Eugenol oil: Eugenol dose (mg) was divided by its specific gravity (1.06) to know its volume to be used in dose preparation. The required volume was emulsified in distilled water by using tween 80 (0.5% of the total volume) (Jaganathan et al., 2010). The emulsion was prepared in such a way that the required dose was contained in 0.4 mL of the emulsion.

Animals and treatment: Forty eight wistar rats of either sex (180-200 g) were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. They were maintained at a constant room temperature (23±2°C) with light-dark cycles of 12:12 h and free access to water and standard laboratory chow. After a 7-day acclimatization period, they were randomly divided into five groups of six each.

All the experimental procedures were carried out in accordance with committee for the purpose of control and supervision of experiments on animal (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.

Rats were divided into 4 groups (n = 12). Acute pancreatitis was induced in these groups by non invasive L-arginine model. L-arginine was given at a dose of 2.5 g kg⁻¹ b.wt. two intraperitoneal injections with an interval of 1 h (Hegyi et al., 2004). Group 1: Served as normal control and received only saline, group 2: Served as disease control and received L-arginine (2.5 g kg⁻¹, i.p., two times with one hour interval)+Vehicle (tween 80), group 3 and 4: Served as test groups and received L-arginine+eugenol 100 and 200 mg/kg/day, p.o. (Daniel et al., 2009; Morsy and Fouad, 2008; Jaganathan et al., 2010), respectively. Treatment was started one hour after the last injection of L-arginine and continued till the day of sacrifice. All the animals received their respective drugs two hour before the sacrifice. In all groups, half of the animals were sacrificed at 24 h and remaining half were sacrificed at 72 h after the last injection of L-arginine. Animals were anaesthetized with anaesthetic ether and blood samples were collected by cardiac puncture for the estimation of serum amylase and lipase and finally the animals were sacrificed for the isolation of pancreas and lungs and stored at -70°C until use.

Pancreatic 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⁻¹) was calculated for each animal, to estimate the level of pancreatic edema (Melo et al., 2010).

Biochemical estimations: Serum amylase and lipase levels were estimated by routine colorimetric methods using the commercial kits (Erba diagnostics, Hyderabad, India) and expressed in U/dl. Pancreatic total protein content was determined using the commercial diagnostic kit (Rapid diagnostics, Hyderabad, India) and expressed in mg g⁻¹ tissue (Sidhu et al., 2010). Malondialdehyde (MDA) level was estimated in pancreas and lungs by the spectroscopic method (Ohkawa et al., 1979) and expressed in nmol/mg protein. Pancreatic catalase activity was measured (Aebi, 1984; Kakkar et al., 1998) and expressed in k/sec/mg protein, where k is the first order rate constant. Superoxide dismutase [SOD] level was measured in pancreas (Misra and Fridovich, 1972; Szaboles et al., 2006) and expressed in U/mg protein. Reduced glutathione [GSH] level was measured in pancreas (Ellman, 1959; Szaboles et al., 2006) and expressed in μM/mL/mg protein.
Histology: Pancreas and lungs were removed immediately and part of it is fixed in 10% neutral buffered formalin and embedded in paraffin by standard methods. Paraffin sections of 5 μm were cut and stained with haematoxylin and eosin and then assessed under dark field microscope and examined blind by a morphologist for grading histopathological changes. Pancreatic damage was assessed and scored by grading edema, inflammation, acinar cell necrosis, haemorrhage and fatty changes (Abdin et al., 2010; Siddhu et al., 2010). Lung damage was assessed and scored by grading edema, haemorrhage, infiltration of neutrophils and alveolar rupture (Brien et al., 2005).

Statistical analysis: Statistical analysis was performed by one way ANOVA followed by Newman-Keuls as post hoc test using GraphPad Prism 5. Values of the measured parameters were presented as mean±SEM. The difference was considered to be statistically significant when p<0.05.

RESULTS

Serum amylase and lipase: As shown in Fig. 1, induction of acute pancreatitis with L-arginine has significantly (p<0.001) increased the serum amylase and lipase levels at 24 h but no significant difference was observed at 72 h when compared to normal control group. Treatment with eugenol (100 and 200 mg kg⁻¹) dose dependently decreased the L-arginine evoked rise in serum amylase and lipase levels at 24 h interval compared to disease control group.

Pancreatic edema and total protein: As shown in Fig. 2, administration of L-arginine significantly (p<0.001)
increased the pancreatic edema and decreased the pancreatic total protein level at 24 and 72 h after the last injection of L-arginine in time dependent manner when compared to normal control group. Treatment with eugenol (100 and 200 mg kg\(^{-1}\)) dose dependently decreased the pancreatic edema and increased the total protein level at both 24 and 72 h time intervals. Administration of eugenol 200 mg kg\(^{-1}\) significantly (p<0.001) reversed the L-arginine evoked rise in pancreatic edema and decline in total protein and brought to normal levels at both time intervals.

**Pancreatic MDA and reduced GSH:** As shown in Fig. 3, induction of pancreatitis resulted in significant (p<0.001) rise in MDA and decline in GSH level at 24 h and more pronounced at 72 h compared to normal control group. Treatment with eugenol (100 and 200 mg kg\(^{-1}\)) dose dependently decreased the MDA and increased the GSH levels at both time intervals when compared to disease control group. Administration of eugenol 200 mg kg\(^{-1}\) significantly (p<0.001) reversed the L-arginine evoked rise in pancreatic MDA and decline in GSH levels and brought to normal levels at both time intervals.

**Pancreatic SOD and catalase:** As shown in Fig. 4, induction of pancreatitis resulted in significant rise in pancreatic SOD and catalase levels at 24 h. Whereas, at 72 h significant fall in SOD and catalase level was observed. Treatment with eugenol (100 and 200 mg kg\(^{-1}\)) dose dependently ameliorated the change in SOD and catalase levels at both time intervals when compared to disease control group. Administration of eugenol 200 mg kg\(^{-1}\) significantly (p<0.001) restored the L-arginine evoked change in SOD and catalase levels and brought to normal levels at both time intervals.

**Lung MDA:** As shown in Fig. 5, induction of pancreatitis resulted in significant rise in lung MDA level at 24 h and more pronouncedly at 72 h compared to normal control group. Treatment with eugenol (100 and 200 mg kg\(^{-1}\))
Fig. 5: Effect of eugenol treatment on lung MDA after L-arginine induced acute pancreatitis. Each column represents mean±SEM (n = 6). *p<0.05 vs. normal control, †p<0.01 vs. normal control, ‡p<0.001 vs. normal control, ‡p<0.05 vs. disease control, †p<0.01 vs. disease control, †p<0.001 vs. disease control, †p<0.001 vs. eugenol 100 mg kg⁻¹, L-arg: L-arginine

Fig. 6(a-b): Representative photomicrographs of pancreatic sections from the normal, L-arginine (L-arg, eugenol and vehicle treated animals (H and E x200), (a): Pancreatic sections at 24 h time point, (b) and Pancreatic sections at 72 h time point; L-arg: L-arginine (arrows represents the pancreatic sections)

dose dependently decreased the lung MDA levels at both time intervals when compared to disease control group. Administration of eugenol 200 mg kg⁻¹ significantly (p<0.001) ameliorated the L-arginine evoked rise in MDA levels at both time intervals.

Pancreatic histology: As shown in Fig. 6 and Table 1, normal pancreatic morphology was observed in normal control group. Moderate edema, haemorrhage, necrosis and inflammation were observed in disease control group at 24 h (Fig. 6a). But by 72 h severe edema, necrosis and inflammation, moderate haemorrhage and fatty change were observed (Fig. 6b). Treatment with eugenol (100 and 200 mg kg⁻¹) has shown dose dependent and time dependent protective effect on pancreas and ameliorated the L-arginine induced histological changes at both
Table 1: Effect of eugenol on total histopathological score of pancreas after L-arginine induced acute pancreatitis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Saline</th>
<th>L-arginine+vehicle</th>
<th>L-arginine+eugenol (100 mg kg⁻¹)</th>
<th>L-arginine+eugenol (200 mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td>Edema</td>
<td>0</td>
<td>0.1</td>
<td>1.5±0.1</td>
<td>1.8±0.01</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>0</td>
<td>0.3</td>
<td>1.7±0.2</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>0.1</td>
<td>0.3</td>
<td>1.2±0.01</td>
<td>1.1±0.01</td>
</tr>
<tr>
<td>Acinar cell necrosis</td>
<td>0.1</td>
<td>0.3</td>
<td>1.6±0.2</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Fatty change</td>
<td>0</td>
<td>0.3</td>
<td>1.6±0.3</td>
<td>0.2±0.1</td>
</tr>
</tbody>
</table>

Data expressed as mean±SEM (n = 6). *p<0.05 vs L-Arginine+vehicle, **p<0.01 vs. L-Arginine+vehicle, ***p<0.001 vs. L-Arginine+vehicle, ****p<0.05 vs eugenol 100 mg kg⁻¹, *****p<0.01 vs. eugenol 100 mg kg⁻¹, ******p<0.001 vs. eugenol 100 mg kg⁻¹

Table 2: Effect of eugenol on total histopathological score of lungs after L-arginine induced acute pancreatitis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Saline</th>
<th>L-arginine+vehicle</th>
<th>L-arginine+eugenol (100 mg kg⁻¹)</th>
<th>L-arginine+eugenol (200 mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td>Edema</td>
<td>0</td>
<td>0.1</td>
<td>1.0±0.2</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>0</td>
<td>0.2</td>
<td>0.6±0.3</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>0.3</td>
<td>0.2</td>
<td>1.0±0.1</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>Avascular collapse</td>
<td>0</td>
<td>0.2</td>
<td>1.8±0.1</td>
<td>0.5±0.2</td>
</tr>
</tbody>
</table>

Data expressed as mean±SEM (n = 6). *p<0.05 vs L-Arginine+vehicle, **p<0.01 vs. L-Arginine+vehicle, ***p<0.001 vs. L-Arginine+vehicle, ****p<0.05 vs eugenol 100 mg kg⁻¹, *****p<0.01 vs. eugenol 100 mg kg⁻¹

Fig. 7(a-d): Representative photo micrographs of lung sections at 72 h interval from (a) Normal, (b) L-arginine+vehicle, (c) L-arginine+eugenol 100 mg and (d) L-arginine+eugenol 200 mg groups (H&E x 200)

Time intervals. Administration of eugenol 200 mg kg⁻¹ significantly (p<0.001) ameliorated the L-arginine induced histiarchitectural changes in pancreas.

**Lung histology:** As shown in Fig. 7 and Table 2, normal lung morphology was observed in normal control group. Mild edema, haemorrhage, neutrophil infiltration and alveolar rupture were observed in disease control group at 24 h (Table 2). But by 72 h severe edema, haemorrhage, neutrophil infiltration and alveolar rupture were observed (Fig. 7). Treatment with eugenol (100 and 200 mg kg⁻¹) has shown dose dependent and time dependent protective effect on lungs and ameliorated the L-arginine induced histoarchitectural changes at both time intervals. Administration of eugenol 200 mg kg⁻¹ significantly (p<0.001) ameliorated the L-arginine induced histoarchitectural changes in lungs.

**DISCUSSION**

The present study demonstrated that treatment with eugenol (200 mg kg⁻¹) efficiently reduced the severity of L-arginine induced acute pancreatitis and associated pulmonary complications in rats. In agreement with the previous studies (Czako et al., 1998; Melo et al., 2010; Sidhu et al., 2010; Tani et al., 1990), in the present study administration of L-arginine significantly developed the acute pancreatitis characterized by elevated levels of serum amylase and lipase at 24 h and severe histoarchitectural changes at 72 h.

L-arginine induced acute pancreatitis is a non-invasive model of acute pancreatitis. Depending on the dose and duration of L-arginine administration different phases of pancreatitis can be studied (Hegyi et al., 2004). High dose of L-arginine significantly affects the acinar cells of pancreas by inhibiting the polyamine synthesis which further inhibits the nucleic acid and protein synthesis, resulting in necrosis (Abdin et al., 2010; Hyvonen et al., 2006). Acinar cells are the first target of L-arginine over dose because protein metabolism is most active in pancreatic acinar cells (Hyvonen et al., 2006; Passaglia, 2007).

Serum amylase and lipase levels are the important diagnostic markers for acute pancreatitis. Which usually
rise within 4-8 h of the initial attack, peaks at 24 h and returns to normal over the 72 h (Czako et al., 1998; Melo et al., 2010). As the pancreatic tissue damage was more severe at 72 h, it leads to marked reduction in pancreatic enzymes production and it might result in lower serum pancreatic enzyme levels regardless of the extent of necrosis (Tani et al., 1990). Similarly, in the present study induction of pancreatitis significantly increased serum amylase and lipase levels at 24 h, whereas these changes were not observed at 72 h. Treatment with eugenol decreased the serum amylase and lipase levels at 24 h, indicated the protective effect of eugenol at early stage of the disease progression.

Involvement of oxidative stress is one of the major causative factors for the initiation and progression of severe acute pancreatitis and associated pulmonary complications (Szabolcs et al., 2006; Czako et al., 1998; Hardman et al., 2005). In agreement with this statement, in the present study significant alteration in MDA, SOD, catalase and GSH levels were observed at 24 h and more pronouncedly at 72 h.

MDA, a marker for lipid peroxidation was elevated in L-arginine treated rats. It could be attributed to the accumulation of free radicals proposed to be generated by L-arginine. Free radicals initiate lipid peroxidation of the membrane bound polyunsaturated fatty acids, leading to impairment of the membrane functional and structural integrity (Szabolcs et al., 2006; Czako et al., 1998; Sidhu et al., 2010). The observed significant increase in SOD and catalase levels indicated that oxidative stress caused by L-arginine may up-regulate the activity of antioxidant enzymes to facilitate rapid removal of accumulated reactive oxygen species (Szabolcs et al., 2006). A significant decrease in SOD and catalase levels at 72 h suggested increased levels of ROS as the disease progressed (Czako et al., 1998). Increased ROS may attack the active site of antioxidant enzymes and make them to lose their function to scavenge ROS. So ROS levels get increased and antioxidant enzyme levels get decreased in chronic disease conditions. GSH is found to be decreased in L-arginine treated rats at 24 h and more pronouncedly at 72 h indicated the enhanced oxidative stress as the disease progressed (Szabolcs et al., 2006).

Changes in the pancreatic and lung MDA, pancreatic edema, catalase, SOD and GSH levels have restored by eugenol administration in time dependent manner at both 24 and 72 h. These findings suggest that treatment with eugenol significantly attenuated the lipid peroxidation and improved the intracellular antioxidant defence in pancreas and lungs.

Passaglia (2007) stated that acinar cells are the protein factory of body. Catabolism of proteins could increase up to 80% in acute pancreatitis and consequently a sharp decline in protein content was observed. In consistent with previous reports (Sidhu et al., 2010) pancreatic total protein content, a marker of tissue damage was found to decrease in L-arginine received rats at 24 h and more pronouncedly at 72 h. Current study revealed that treatment with eugenol significantly increased the total protein content at both 24 and 72 h.

The extent of damage to pancreas and lung tissues in acute pancreatitis correlates with the level of free radical generation. In accordance with previous studies (Czako et al., 1998; Melo et al., 2010; Abdin et al., 2010; Sidhu et al., 2010; Hardman et al., 2005; Brien et al., 2005), in the present study histopathological assessments revealed that induction of pancreatitis resulted in time dependent damage to pancreas and lungs. Treatment with eugenol ameliorated the histoarchitectural changes in both pancreas and lungs.

CONCLUSION

In conclusion, the present study suggests that treatment with eugenol significantly ameliorated the L-arginine induced pancreatitis and associated pulmonary complications probably due to its antioxidant property. However, further studies are needed to evaluate molecular level mechanism of eugenol and its clinical usefulness.

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