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Protective effect of lawsone on L-Arginine induced acute pancreatitis in rats

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The efficacy of lawsone against L-arginine induced acute pancreatitis was determined at 24 h by determination of serum levels of amylase, lipase and proinflammatory cytokines [tumor necrosis factor (TNF)-α, C-reactive proteins and interleukin (IL)], pancreatic myeloperoxidase (MPO) activity, lipid peroxidation (thiobarbituric acid reactive substances (TBARS)], nitrate/nitrite levels, and the wet weight/body weight ratio. Lawsone and methylprednisolone treatments significantly attenuated the L-arginine- induced increases in pancreatic wet weight/body weight ratio, and decreased the serum levels of amylase and lipase, and TNF-α and IL-6 and significantly lowered pancreatic levels of MPO, TBARS, and nitrate/nitrite. The histoimmunological findings further proved the amelioration of pancreatic injury by lawsone and further proved anti-inflammatory and antioxidant agent property of lawsone.

Keywords: Acute pancreatitis, Anti-inflammatory, Cytokines, L-arginine, Lawsone

Inflammation of pancreatic gland called pancreatitis (AP), may leads to sever complication and high mortality without treatment. The pathogenesis is not fully understood, however the leukocyte activation, microcirculatory disturbances and oxidative stress are the major constituents of AP. This is characterized by activation of widespread inflammatory cell infiltration, leukocyte and digestive proteases. Further, by releasing reactive oxygen, nitrogen species and various kinds of inflammatory mediators. 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]. No specific treatment is available to treat AP. Many therapies and medical management is aimed to control the sign and symptoms of AP, using steroids, analgesics and anti-inflammatory agents. The use of the synthetic and semi-synthetic treatment has various kinds of drawbacks like photosensitivity skin reactions, intolerance and addiction. Apart from this, these compounds 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 and anti-carcinogenic activities of lawsone have been reported[3,4]. The lawsone has haematotoxic properties, leading to stimulation of cell proliferation; representing a sufficient explanation for a weak induction of 'late micronuclei'. Hence in this study the non-toxic dose of lawsone (100 and 200 mg/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 180-200 g obtained from Mahaveer Enterprises, Hyderabad were maintained at a constant room temperature (23±2 °C) with 12:12 h light-dark cycles and free access to water and standard laboratory chow. The rats were randomly divided into 6 groups of 8 in each and experiments were performed after 12 h 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

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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), lawsone (Sigma Aldrich Co Pvt Ltd, Japan), 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 500 mg/mL and the *p*H was adjusted to 7 with 5 *N* HCl.

Lawsone was prepared as a solution by dissolving in 3% tween 80 and 0.9% saline to a final concentration of 100 and 200 mg/mL and the *p*H was adjusted to 7 with 0.1 *N* NaOH.

L-arginine-induced pancreatitis model—Acute pancreatitis was induced in five groups of rats by two intraperitoneal (ip) injections of L-arginine (2.5 g/kg, 1 h apart). One hour following the last injection of L-arginine, the rats were treated orally as follows: Gr. 1 received the vehicle (3% Tween 80) of lawsone (vehicle control); Grs 2 and 3 were treated with lawsone (100 and 200 mg/kg, respectively). Gr. 4 acted as positive control and received methylprednisolone (30 mg/kg), all in a volume of 10 mL/kg and Gr. 5, received saline (0.9%, NaCl, ip) in place of L-arginine and served as a normal control. After 24 h 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[5].

Serum analysis—For serum analysis, blood samples were centrifuged at 3000 *g* at 4 °C for 10 min. The serum amylase and lipase were determined by routine colorimetric methods using the commercial kits for amylase (Rapid diagnostics), lipase (Accurex diagnostics), C-reactive protein and interleukin-α and expressed as U/dl[5].

Biochemical estimations—Pancreatic total protein content[6] was determined. Myeloperoxidase activity[7], nitrate/nitrite level[8,9], TBARS level[10], catalase activity[11,12], SOD level[13] and reduced GSH level[14] 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 5 µm 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[15,16] 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 lawsone (100 and 200 mg/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 lawsone (100 and 200 mg/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 lawsone (100 and 200 mg/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 lawsone (100 and 200 mg/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 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 lawsone (100 and 200 mg/kg) and methyl prednisolone (30 mg/kg) ameliorated the inflammation, edema and more significantly acinar cell degeneration and necrosis and protected the pancreas from L-arginine induced damage. Treatment with lawsone dose dependently decreased the total pathological scores compared to disease control group.

Table1—Effect of lawsone on pancreas weight, total body weight, serum amylase, serum lipase, total nitrate, total protein, MDA, MPO and SOD after L-arginine induced acute pancreatitis
[Values are mean ± SE from 6 animals in each group]

Parameter/Groups	NC	DC	STD	LW 100	LW 200
Pancreas weight	15.36±870.3	19.04± 1015*	18.83±911.3α	46.33± 843.3α	21.37±788.3α
Total body wt	4.889±187.5	3.578 ± 192*	3.60 ± 191.2	5.57 ± 188.7	3.68 ± 189
Serum Amylase	2000±85.63	7667±349*	3317±110.8α	2733±140.6α	3167±187.4α
Serum Lipase	191.7±4.014	566.7±30.84*	346.7±39.21α	236.7±14.06α	260±7.303α
Total Nitrate	1.372±11.87	1.462±16.07*	0.39 ± 7.06α	1.31 ± 10.13α	0.77 ± 8.5α
Total Protein	0.032± 0.73	0.037±0.355*	0.067 ± 0.91α	0.02 ± 0.73α	0.05 ± 0.82α
Kidney GSH	0.011 ± 0.47	0.04± 0.284 *	0.014 ± 0.71α	0.017 ± 0.51β	0.049 ± 0.64α
Liver GSH	0.010 ± 0.48	0.04 ±0.284 *	0.03 ± 0.67α	0.03 ± 0.48 β	0.04 ± 0.59α
Lung GSH	0.020 ± 0.50	0.04± 0.284 *	0.03 ± 0.67α	0.02 ± 0.51γ	0.04 ± 0.60 β
Pancreas GSH	0.011 ± 0.48	0.04± 0.284 *	0.03 ± 0.67α	0.02 ± 0.52 β	0.04 ± 0.57α
Kidney MDA	0.92 ± 13.91	1.04± 17.09 *	0.52 ± 9.61β	0.68 ± 12.31α	0.39 ± 10.72α
Liver MDA	0.88 ± 14.36	1.04 ±17.09 *	0.62 ± 10.96γ	0.85 ± 13.97α	0.62 ± 12.56c
Lung MDA	0.66 ± 14.95	1.04 ± 17.09	1.04 ± 9.82β	1.70 ± 13.18α	0.66 ± 11.5α
Pancrease MDA	1.62±0.6615	1.04±0.4267*	2.39±0.9756α	1.70±0.69 β	1.21±0.49 β
Pancreas MPO	4.75 ± 2.1	32.8 ± 4.7*	6.2 ± 2.2α	17.2 ± 3.7α	11.2 ± 3.7α
Lung MPO	8.3 ± 2.4	43.7 ± 7.1*	11.1 ± 3.0α	28.4 ± 3.3α	13.4 ± 3.3g
Liver MPO	7.75 ± 2.1	33.8 ± 4.7*	9.2 ± 2.2α	22.2 ± 3.4α	14.2 ± 3.8α
KIdney MPO	5.3 ± 2.4	39.7 ± 7.1*	8.2 ± 3.0α	26.4 ± 3.5α	13.4 ± 3.6α
Pancreas Catlaze	0.33±0.13	0.41±0.1707*	0.19±0.07	0.23±0.0934β	0.23±0.0948α
kidney SOD	2.33 ±0.95	1.50±0.6146	2.61±1.06α	1.89±0.774	1.891±0.771β
Liver SOD	0.83±0.34	1.01±0.4153	1.97±0.80α	1.527±0.62β	1.78±0.7266β
Lung SOD	1.04±0.42	1.03±0.42*	3.44±1.40α	2.06±0.84	3.83±1.563α
Pancrease SOD	2.09±0.85	1.51±0.6191*	1.49±0.61 α	1.32±0.54	1.81±0.74

*P < 0.0001 when compared with normal control, αp< 0.0001, βp< 0.001, γp< 0.01 when compared with disease control group

Table 2—Effect of lawsone on Interleukins values, C - reactive protein and TNF- α
[Values are mean ± SEM from 6 animals in each group]

Serum parameters	NC	DC	LW1	LW2
Interlukin-6	29.3±1.569	90.48±1.689*	62.42±1.358γ	33.57±1.182γ
TNF-α	19.33±1.541	26.32±3.036	21.58±0.9711	18.57±1.316
C-Reactive protein	415.3±7.762	16403±119*	8611±101.7γ	546.8±9.874 γ

N.C- Normal Control, D.C- Disease Control, LW 100- Lawsone 100 mg/kg, LW 200- Lawsone 200 mg/kg. *P < 0.0001 when compared with normal control, γP< 0.01 when compared with disease control group.

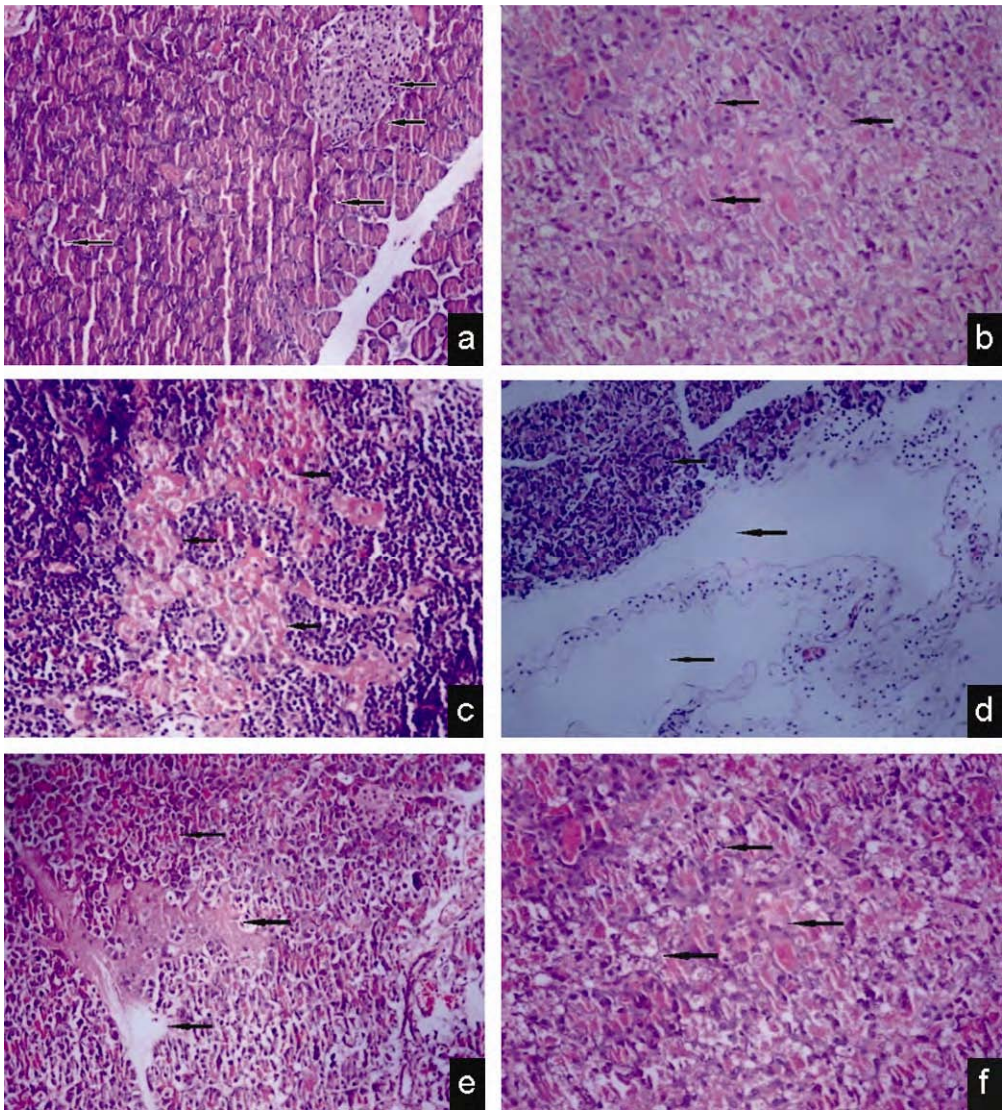

Fig. 1—Effect of lawsone on pancreatic histopathological changes after L-arginine induced acute pancreatitis [(a) normal control, (b) standard control, (c) disease control 24 h hemorrhage, (d) disease control 24 h edema and necrosis, (e) lawsone 100 mg/ kg, ip, 24 h, (f) lawsone 200 mg/ kg, ip, 24 h] (H&E ×200)

Discussion

The present study demonstrated that treatment with lawsone (200 mg/kg) efficiently reduced the severity of L-arginine induced acute pancreatitis in rats. In consistent with previous reports[2,17,18], in the present study administration of L-arginine significantly developed the acute pancreatitis characterized by raised levels of serum amylase, lipase and acinar cell necrosis.

Serum amylase and lipase levels are the important diagnostic markers for acute pancreatitis. They usually rise within 4–8 h of the initial attack, peaks at 24 h and returns to normal over the 72 h[5,6,17]. Similarly, in accordance with previous reports, in the present study induction of pancreatitis significantly increased the serum amylase and lipase levels at 24 h. Treatment with lawsone decreased the serum amylase and lipase levels, indicating protective effect of lawsone at early stage of the disease progression.

In consistent with previous reports[5,6,17], in the present study induction of pancreatitis significantly increased the pancreatic MPO, MDA, nitrite, catalase and SOD and decreased the GSH levels. MPO, a marker of neutrophil infiltration is an enzyme found in neutrophils and its activity is linearly related to infiltration of neutrophils[19,20]. In agreement with previous reports[5,21], induction of pancreatitis with L-Arginine increased the pancreatic MPO levels. Inhibition of the neutrophil infiltration can attenuate

the pancreatic injury[22]. Treatment with lawsone significantly decreased the pancreatic MPO levels probably due to its anti-inflammatory action.

MDA, a marker of lipid peroxidation was 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[6,17,23] 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[23] reported the fall in these enzyme levels at 24 h. However, Szabolcs *et al.*[17] 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[17]. It is well known that GSH is found to be decreased in L-arginine treated rats indicating enhanced oxidative stress as the disease progresses[17].

The role of NO in the initiation and progression of acute pancreatitis remains controversial[24]. Some studies[25-28] reported that NO increase the pancreatic blood flow and/or secretion in response to endothelium derived NO and ameliorates the pancreatic dysfunction, others[29-31] suggested that NO aggravates pancreatic oxidative stress and damage. In agreement with previous reports[31], in the present study significant increase in NO and pancreatic edema was observed in L-arginine received rats. Takacs *et al.*[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.

Treatment with lawsone 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 Sidhu *et al.*[6], pancreatic total protein content, a marker of the tissue damage was found to decrease in L-arginine received rats. Treatment with lawsone 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[5,6,21], 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 lawsone protected the pancreas from L-arginine induced injury. In conclusion, the present study suggests that treatment with lawsone 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 lawsone.

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The views and opinions expressed in this article are those of the authors, and they do not reflect in any way those of the institutions to which they are affiliated with.

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