Journal of Animal Research: v.9 n.5, p. 767-773. October 2019

DOI: 10.30954/2277-940X.05.2019.21

Ameliorating Effect of Melatonin on L- Arginine Induced Acute Pancreatitis in Rats

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Received: 14 Sept., 2019 **Revised:** 25 Sept., 2019 **Accepted:** 30 Sept., 2019

ABSTRACT

The present study was aimed to evaluate the ameliorative potential of melatonin against L-arginine induced acute pancreatitis in rats. Male Sprague Dawley rats (150–240 g) were divided into 3 groups, viz. group I (control group), group II (acute pancreatitis control group) and group III (treatment control) which were further subdivided into 3 subgroups according to time points of 24 hours, 3 days and 7 days. Rats from groups II and III received two injections of L-arginine (2 g/kg i.p.) at 1 h intervals for induction of acute pancreatitis. Melatonin was administered to group III daily at a single dose of 10 mg/kg i.p. On 6 hours, 24 hours, 3 days and 7 days, blood samples were obtained from each group and subjected for the assays of oxidative stress and serum biochemical parameters. Erythrocytic lipid peroxides contents in acute pancreatitis group were significantly higher, while reduced glutathione contents were significantly lower in comparison with the normal controls. The activities of other antioxidant enzymes were also significantly low in these rats. Moreover, significantly increased activities of serum amylase and serum lipase were found in these rats. Administration of melatonin significantly reduced the over production of malonaldialdehyde levels. Other antioxidant enzymes viz. reduced glutathione, superoxide dismutase and catalase activities were improved significantly in melatonin treated rats. Melatonin had also considerably ameliorated the altered serum amylase and serum lipase levels towards normalcy. Thus, it can be concluded that melatonin may possess therapeutic efficacy against L-arginine induced acute pancreatitis in rats.

Keywords: Acute pancreatitis, melatonin, L-arginine, oxidative parameters

Acute pancreatitis is a serious inflammatory disease of the exocrine pancreas associated with the development of necrotizing pancreatitis with systemic complications like systemic inflammatory response syndrome, disseminated intravascular coagulation and multi-organ failure (Uhl, 2011; Petrov *et al.,* 2010). It occurs when digestive enzymes are activated prematurely within the acinar cells of pancreas causing auto digestion resulting in inflammation and necrosis of pancreatic and peripacreatic tissue. A number of pro-inflammatory cytokines released by pancreatic cinar cells, endothelial cells, neutrophils, lymphocytes, and macrophages during acute pancreatitis (Malleo *et al.,* 2007) play an important role in exacerbating the pancreatic tissue inflammation and related systemic complications (Makhija and Kingsnorth, 2002). Many studies have drawn special attention to crucial role of oxidative stress in pathogenesis of acute pancreatitis.

There is no specific treatment known that can alter the course of the disease and the treatment of acute pancreatitis remains supportive (Baker, 2004). Search for novel specific therapeutic approaches for managing acute pancreatitis is ongoing. Currently increased emphasis is on limiting the damage in acute pancreatitis via ameliorating effect of inflammatory mediators. Due to its anti-inflammatory properties (Jaworek *et al.,* 2007) and potent antioxidant properties (Ochoa *et al.,* 2011) together with its high bioavailability owing to its high lipophilicity (Reiter

How to cite this article: Radhika, Kumar, M., Thakur, N., Singh, A., Ilyas, W., Singh, A.K. and Shukla, S.K. (2019). Ameliorating effect of melatonin on L- arginine induced acute pancreatitis in rats. *J. Anim. Res.,* **9**(5): 767-773.

et al., 2007); melatonin has attracted much attention among researchers as a promising therapeutic agent in experimental models of pancreatitis. Melatonin and its receptors are present in the pancreatic gland (Stebelová *et al.,* 2010) and melatonin plays a role in protection of pancreas from inflammatory injury in acute pancreatitis (Jaworek *et al.,* 2007).

The present study was designed to evaluate effect of melatonin on reducing severity of L- arginine induced acute pancreatitis in rats by using pancreatic enzymes and oxidative parameters related to pancreatic injury caused by L- arginine induced pancreatitis in rats.

MATERIALS AND METHODS

Ethical approval

The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC), GBPUAT, Pantnagar vide approval number IAEC/VMD/CVASc/331 dated 14/05/2018 and the study was performed in compliance with the guidelines laid out by the Institutional Animal Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Experimental design

Male Sprague Dawley rats (150–240 g) procured from Experimental Animal Facility of Central Drug Research Institute, Lucknow, U.P. were divided into 3 groups, viz. group I (control group), group II (acute pancreatitis control group) and group III (treatment control) which were further subdivided into 3 subgroups according to time points of 24 hours, 3 days and 7 days. Each subgroup consisted of 5–6 animals. After acclimatization for two weeks, the rats were fasted for 12 hours with free access to water before the induction of acute pancreatitis. Rats from groups II and III received two injections of L-arginine (Sigma-Aldrich, USA) (2 g/kg i.p.) in 0.9% sodium chloride (pH 7.0) at one hour intervals for development of acute pancreatitis (Hegyi *et al.,* 2004). The control group received the same volume of 0.9% sodium chloride. Melatonin (Sigma-Aldrich, USA) was used as a positive treatment agent. It was administered daily at a single dose of 10 mg/kg i.p. after 2 h of L-arginine injection till the day of sacrifice to rats of group III (Sidhu *et al.*, 2010).

Blood samples were collected from retro-orbital plexus at 24 hours, day 3 and day 7 for analyzing biochemical and oxidative parameters.

Biochemical analysis

Estimation of serum enzymes, *viz.* amylase and lipase was done by commercially available kits (Erba Diagnostics Transasia, Mumbai) using protocol supplied with the respective kits and values were expressed in U/l.

Evaluation of oxidative stress indices in erythrocytes

Preparation of red blood cells (RBCs) suspension and RBC hemolysate

Blood samples collected in tubes containing heparin (10 IU/ml of blood) were centrifuged to harvest the erythrocytes. Erythrocytes were washed thrice in the ice-cold isotonic sodium chloride solution (NSS) and were diluted with ice-cold distilled water in 1:10 ratio for the preparation of 10% hemolysate and rest of the RBC pellet was diluted with ice-cold NSS in 1:1 ratio to get RBC suspension which was used for GSH estimation. The prepared 10% hemolysate was used for estimation of superoxide dismutase (SOD), lipid peroxidation (LPO) and catalase (CAT). Hemolysate and RBC suspension were kept at -20°C and used for assay within 6 hours. All assessments were performed in triplicate.

Reduced glutathione (GSH)

Reduced glutathione was estimated by 5, 5-dithiobis-(2-nitro- benzoic acid) (DTNB) method of Prins and Loos (1969) in RBC suspension. Reduced glutathione concentration in the test sample was calculated by employing the molar extinction coefficient of DTNB-GSH conjugate (ηmol/mg Hb), 13600/M/cm.

Lipid peroxidation (LPO)

The lipid peroxides level in the RBC hemolysate was determined by the method of Placer *et al.* (1966) in terms of concentration of malonaldialdehyde (MDA), a marker of lipid peroxidation. The concentration of MDA in μmolmg^{-1} of RBC hemolysate was calculated using the

extinction coefficient of 1.56×10^{-5} L $mmol^{-1}cm^{-1}$.

Catalase (CAT)

Catalase activity in hemolysate was estimated using H_2O_2 as a substrate as per the method of Bergmayer (1983). One unit of activity is equal to mmoL of H_2O_2 degraded per min and was expressed as units/mg of Hb.

Superoxide dismutase (SOD)

Superoxide dismutase was estimated as per the method described by Madesh and Balasubramanian (1998). SOD was expressed as SOD units/ mg of haemoglobin.

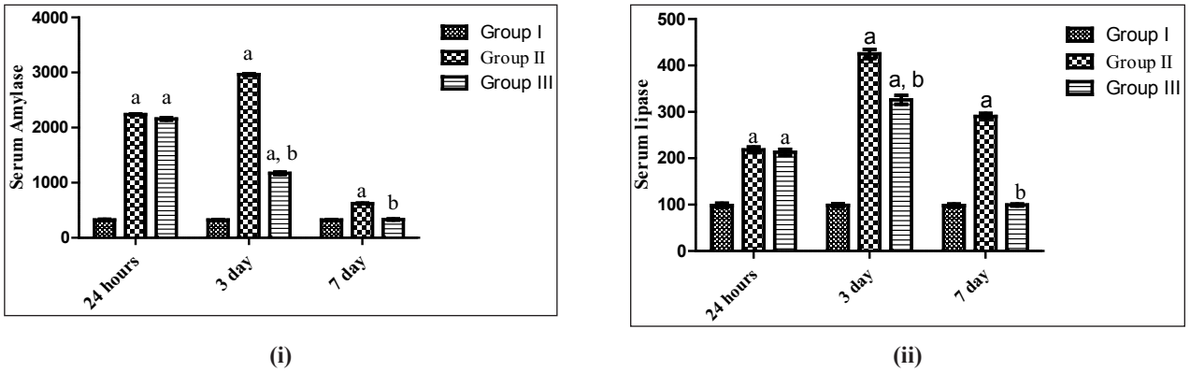
STATISTICAL ANALYSIS

The data obtained were analyzed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad PRISM™ software (Graph Pad Software Inc., CA, USA). The p value of less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Administration of L-arginine significantly increased serum amylase levels in rats of group II and group III at 24 hours (2238.83 ± 14.38 u/l), (2156.67 ± 23.33 u/l) and at 3 days (2964.67± 15.47 u/l), (1173± 23.46 u/l) in comparison with healthy control ($p<0.001$) (Table 1). However, melatonin treatment showed significant reduction in serum amylase at day 3 (1173± 23.46 u/l) and day 7 (332.67± 12.39 u/l) interval in comparison with group II ($p<0.001$) indicating its beneficial effect in experimental acute pancreatitis. L-arginine-induced pancreatitis caused a significant increased serum lipase levels at 24 hours (218.33± 6.01 u/l) and day 3 (425± 9.62 u/l) as compared with healthy control rats ($p <0.001$). Melatonin treatment significantly lowered the serum lipase levels at day 3 (213± 6.46 u/l) and day 7 (99.5± 2.16 u/l) when compared with group II animals ($p <0.001$) (Fig. 1).

Erythrocytic GSH contents were significantly lower in acute pancreatitis control (group II) (15.33 ± 1.33 ηmol/mg Hb) at 24 hour and at 3 days (19.33 ± 1.77 ηmol/mg Hb) interval in comparison with the normal control. However melatonin treatment significantly improved erythrocytic GSH contents at 24 hours (30.33 ± 0.95 ηmol/mg Hb) and 3 day (30.17 ± 2.86 ηmol/mg Hb) in comparison to acute pancreatitis control (group II) indicating its strong antioxidant potential (Table 1 and Fig. 2). The marker of lipid peroxides (LPO), MDA contents in erythrocytes of acute pancreatitis control (group II) were significantly higher at 24 hours (0.58 ± 0.04 µmol MDA/mg Hb) and 3 day interval (0.60 ± 0.03 µmol MDA/mg Hb) in comparison with the normal control (group I). Melatonin treatment significantly decreased MDA contents at 24 hours (0.41± 0.03 µmol MDA/mg Hb) and 3 day (0.39 ± 0.03 µmol MDA/mg Hb) in comparison to acute pancreatitis control (group II). Levels of erythrocytic antioxidant enzyme catalase (CAT) were also found to be significantly reduced in acute pancreatitis control (group II) (105.67± 4.88 K/



(i) (ii)

Fig. 1: Effect of melatonin on serum amylase and lipase in L- arginine induced acute pancreatitis in rats

Values are expressed as mean ± SEM. [a]$p<0.05$ compared with normal control group (group I), [b]$p<0.05$ compared with L-arginine control group (group II).

Table 1: Effect of melatonin on serum biochemicals and oxidative stress indices in erythrocytes in L- arginine induced acute pancreatitis in rats

Parameter	Group I			Group II			Group III		
	24 h	**3 d**	**7 d**	**24 h**	**3d**	**7d**	**24 r**	**3 d**	**7d**
Serum amylase (U/L)	325.33 ± 11.82	324.33 ± 10.19	325 ± 9.85	2238.83 ± 14.^{3}8a	2964.67 ± 15.^{4}7a	622. 33 ± 8.^{0}9a	2156.67 ± 23.^{3}3a	1173 ± 23.^{6a}6b	332.67 ± 12.^{9}9b
Serum lipase (U/L)	98.33 ± 4.89	98.67 ± 3.56	98 ± 3.26	218.33 ± 6.0^{1}a	425 ± 9.6^{2}a	290.33 ± 6.3^{5}a	213 ± 6.4^{6}a	326 ± 9.^{7a}b	99.5 ± 2.16^{b}
GSH (ηmol/mg Hb)	31.67 ± 2.39	31.5 ± 2.28	31.67 ± 2.10	15.33 ± 1.33^{a}	19.33 ± 1.77^{a}	26.33 ± 2.85	30.33 ± 0.95^{b}	30.17 ± 2.86^{b}	30.33 ± 1.82
LPO (µmol MDA/mg Hb)	0.41 ± 0.02	0.41 ± 0.02	0.40 ± 0.04	0.58 ± 0.04^{a}	0.60 ± 0.03^{a}	0.46 ± 0.04	0.41 ± 0.02^{b}	0.39 ± 0.03^{b}	0.42 ± 0.03
CAT (K/mgHb)	163.67 ± 3.63	162 ± 4.44	161.67 ± 4.28	105.67 ± 4.88^{a}	120.33 ± 6.48^{a}	153 ± 5.40	145.17 ± 7.62^{b}	149.33 ± 8.08^{b}	158.5 ± 5.6
SOD (U/mg Hb)	1.16 ± 0.11	1.15 ± 1.14	1.14 ± 0.06	0.47 ± 0.1^{a}	0.58 ± 0.06^{a}	1.1 ± 0.17	1.04 ± 0.89^{b}	1.03 ± 0.10^{b}	1.11 ± 0.06

Values are expressed as mean ± SEM. $^{a}p<0.05$ compared with normal control group (group I), $^{b}p<0.05$ compared with L-arginine control group (group II).

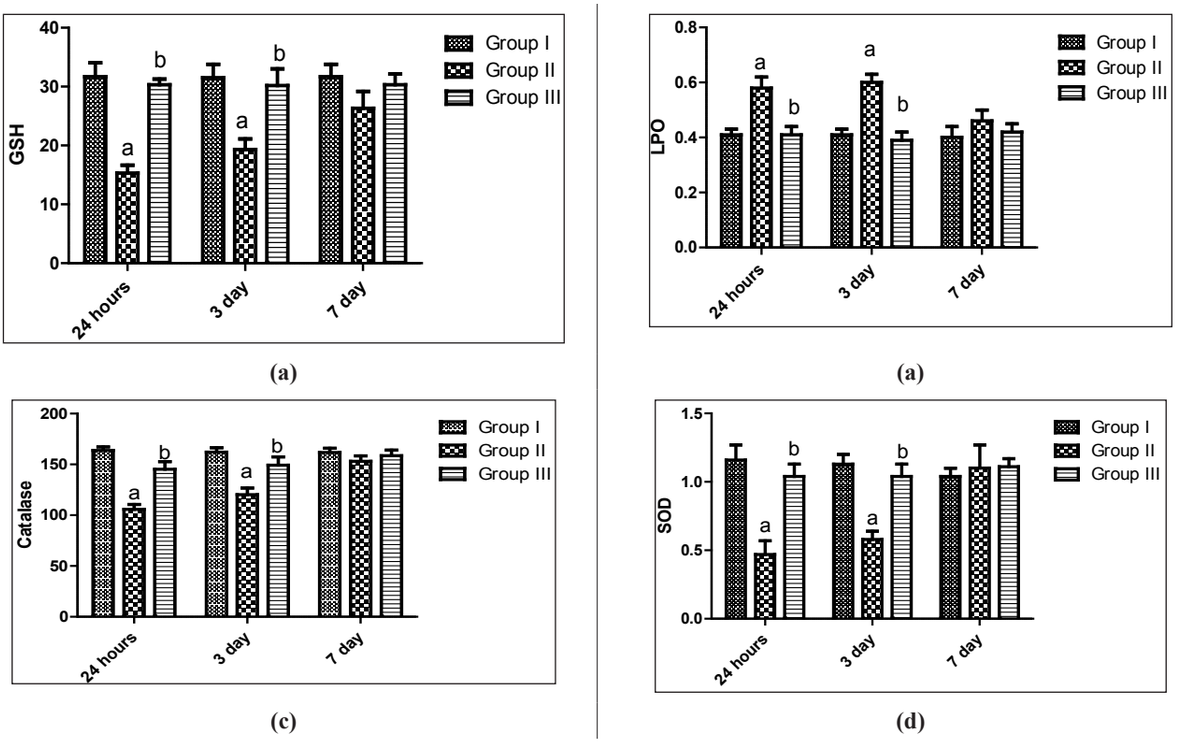


Fig 2: Effect of melatonin on oxidative stress indices in erythrocytes in L- arginine induced acute pancreatitis in rats

Values are expressed as mean ± SEM. $^{a}p<0.05$ compared with normal control group (group I), $^{b}p<0.05$ compared with L-arginine control group (group II).

mg Hb) at 24 hour and at 3 days interval (120.33± 6.48 K/mg Hb) in comparison with the normal control. Melatonin treatment significantly increased CAT levels at 24 hours (145.17± 7.62 K/mg Hb) and 3 day interval (149.33± 8.08 K/mg Hb) when compared with acute pancreatitis control. The content of erythrocytic SOD was found to be significantly reduced in acute pancreatitis control in comparison to normal control at 24 hours (0.47± 0.1 U/mg Hb) and 3 day (0.58 ± 0.06 U/mg Hb) interval (Table 1 and Fig. 2). Melatonin treatment significantly raised SOD content at 24 hours (1.04± 0.89 U/mg Hb) and 3 day (1.03 ± 0.10 U/mg Hb) as compared with the acute pancreatitis control group.

Present study exhibited protective effect of melatonin on reducing severity of L-arginine induced acute pancreatitis in rats by markedly decreasing stress. Administration of L-arginine produced time dependent effects on the pancreas as manifested by increasing levels of serum biochemical markers, viz. amylase and lipase after L-arginine administration reaching its peak at 3 day post administration. Protective effect of melatonin in reducing the severity of acute pancreatitis was evidenced as post treatment with melatonin lead to significant decrease in serum amylase and lipase in comparison with acute pancreatitis control. Moreover, treatment with melatonin was able to restore serum amylase and lipase levels towards normal range after 7 days of treatment (Fig. 1) illustrating its therapeutic efficacy in L-arginine induced acute pancreatitis in rats. Although the exact mechanisms of protective effect of melatonin in acute pancreatitis are not precisely known, however the proposed probable mechanisms includes scavenging of the oxygen radicals, activation of various antioxidant enzymes (Rodriguez *et al.,* 2004), reduction of inflammation (Carrillo-Vico *et al.,* 2005) and increase of regeneration in the pancreas (Jaworek *et al.,* 2014).

The role of oxygen derived free radicals in the pathogenesis of acute pancreatitis has been documented by many workers (Guice *et al.,* 1986; Pérez *et al.,* 2015). A reliable measure of assessing the involvement and extent of oxygen-radical-induced tissue damage is difficult due to their high reactivity. Therefore to measure oxidative tissue damage, measurement of the effects of radical reactions with biological substances (i.e. lipid peroxides and/or their decomposition products), and treatment with scavengers which detoxify oxygen radicals has been proposed (Schoenberg *et al.,* 1992). The increase in malondialdehyde (MDA) levels have been associated with tissue injury in acute pancreatitis (Hernández *et al.,* 2011) and increased levels of MDA have also been related with pancreatitis associated multiple organ dysfunction (Shi *et al.,* 2005). Glutathione plays an important role in protecting cells against oxidative stress (Zollner *et al.,* 1991) and increase of cellular glutathione indicate a higher exposure of erythrocyte to the risk of oxidative stress (Ohtsuka *et al.,* 1997; John *et al.,* 2001). GSH depletion has been considered a hall mark during the initial phase of acute pancreatitis (Neuschwander-Tetri *et al.,* 1992) as GSH depletion allows a premature activation of digestive enzymes inside acinar cells triggering the inflammatory process (Schulz *et al.,* 1999). Antioxidant enzymes like SOD and catalase aid in reducing the ultrastructural and biochemical injury associated with acute pancreatitis in rats (Guice *et al.,* 1986).

The results of the present study positively indicate that L-arginine induced acute pancreatitis mediates oxidative stress as significant elevation in LPO (MDA) levels and reduction in GSH, SOD and CAT levels in rat erythrocytes was noticed after L-arginine administration. Overproduction of free radical in acute pancreatitis group rats might have upshot the erythrocytic lipid peroxidation resulting in increased MDA contents. Oxidative stress induced in L-arginine induced acute pancreatitis reflected with diminished erythrocytic GSH, SOD and CAT contents, because of exhaustion of GSH, SOD and CAT stores to combat increased number of free radicals produced.

Many studies have shown antioxidant potential of melatonin and mechanisms suggested for its antioxidant activity includes free radical scavenging, stimulation of antioxidative enzymes, increasing the efficiency of mitochondrial oxidative phosphorylation (Rodriguez *et al.,* 2004). In the present study, post treatment with melatonin in L-arginine induced acute pancreatitis ameliorated increased MDA content towards normalcy (Fig. 2) suggesting its potential in reducing lipid peroxidation mediated oxidative damage. Also, melatonin treatment was associated with increased erythrocytic antioxidant enzymes, viz. GSH, SOD, CAT in comparison to acute pancreatitis control. Results of present study suggest that attenuation of oxidative stress by treatment with melatonin may be responsible for amelioration of L-arginine induced

acute pancreatitis as oxidative injury play key role in sustaining the pancreatic damage in pancreatitis.

CONCLUSION

Oxidative stress plays a crucial role in patho-physiology of acute pancreatitis. Prophylactic and therapeutic efficacy of antioxidants has been demonstrated by substantial research done on acute pancreatitis in various animal models. Present study suggested that the post-treatment of melatonin has a potential antioxidant action to restore the erythrocytic antioxidants stores in L-arginine induced acute pancreatitis in rat model. These results warrant further investigation of melatonin as a potential therapeutic agent not only against acute pancreatitis but the ailments where marked oxidative stress is manifested.

ACKNOWLEDGEMENTS

We are highly thankful to the Director, Experiment Station, Dean, Post-graduate Studies and Dean, College of Veterinary & Animal Sciences of the University for providing the necessary facilities. The first author is also indebted to Indian Council of Agriculture Research, New Delhi for awarding Senior Research Fellowship during the Ph.D.

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