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Acetyl-L-Carnitine Modulates Bleomycin-Induced Oxidative Stress and Energy Depletion in Lung Tissues

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ABSTRACT

Background and Purpose: The usefulness of Bleomycin (BLM) as an important antineoplastic drug is usually limited to the development of dose and time-dependent interstitial pneumonitis and pulmonary fibrosis. This study has been initiated to investigate the possible protective effects of acetyl-L-carnitine (AC) against BLM-induced lung toxicity at an early stage of its development.

Material and Methods: A total of 40 male Sprague-Dawley rats weighing from 200-250g each, were divided into 4 groups of 10 animals each. The first group received a daily i.p. injection of normal saline (0.5ml/200gm body weight) for 5 consecutive days and served as a control. Animals in the second, third and fourth groups were daily injected intraperitoneally (i.p.) with BLM (15mg/kg body weight), AC (250mg/kg body weight) and AC (250mg/kg) 2 hrs before BLM (15mg/kg) each for 5 consecutive days, respectively.

Results: Treatment of rats with BLM (15mg/kg) resulted in a significant 3.4 and 2.9 folds increase in malondialdehyde (MDA) and nitric oxide (NO) production in lung tissue, respectively and a significant 39%, 35%, 54% and 44% decrease in reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and adenosine triphosphate (ATP), respectively as compared to the control group. Treatment of rats with AC did not lead to any significant change in the mentioned biochemical parameters in the lung tissue. Administration of AC two hours before BLM attenuated BLM-induced increase in MDA and NO and the decrease in GSH, SOD, GSHPx and ATP in lung tissue.

Conclusion: The present data suggests that the protective effect of AC against BLM-induced acute lung injury could be, at least in part, due to its free radical scavenging properties with the consequent improvement in mitochondrial function and ATP production.

Key Words: *Bleomycin - Acetyl-L-carnitine - Lung toxicity - Oxidative stress - Adenosine triphosphate.*

INTRODUCTION

Bleomycin, a glycopeptide antibiotic, is an anticancer drug with broad-spectrum antineoplastic activity against different human tumors including cancers of head and neck, oesophageal carcinoma, malignant lymphomas, testicular carcinoma and malignant pleural effusions **[1]**. BLM has a wide range of toxicity and the lungs are the main target organ for its adverse effects. The optimal clinical usefulness of BLM in cancer chemotherapy is usually limited due to the development of dose and time-dependent interstitial pneumonitis and pulmonary fibrosis **[2]**. The manifested lung toxicity of BLM is greatly affected by the total dose of the drug, the route of its administration and the age of the cancer patient **[3]**. The cause of BLM-induced pulmonary toxicity is not fully explored and seems to be multifactorial. Several mechanisms have been proposed, including a rise in the level of tumor necrosis factor-alfa, generation of reactive oxygen species such superoxide anion and hydroxyl radicals **[4-7]**, stimulation of endothelial cell transforming growth factor-B1 **[8,9]**, depletion of NAD (nicotinamide dinucleotide) and ATP **[10]**, DNA damage and apoptosis in the lung **[11]**, and the possible involvement of platelet activating factor **[12,13]**.

Acetyl-L-carnitine is a naturally occurring short chain derivative of L-carnitine which is synthesized endogenously in human brain, liver and kidney by the enzyme acetyl carnitine transferase or obtained from dietary sources **[14]**. L-carnitine and its short chain derivatives are essential cofactors for mitochondrial transport

and oxidation of long chain fatty acids and also act as scavengers of oxygen free radicals in mammalian tissues **[15]**. AC facilitates the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation, enhances acetylcholine production, and stimulates protein and membrane phospholipids synthesis. AC has antioxidant activity towards oxidative stress via an inhibition of the increase in lipid hydroperoxidation **[16]**. Recently, Arafa and Sayed-Ahmed **[17]** reported that AC offered complete protection against alcohol-induced gastric lesions in rat secondary to its anti-radical effects. Owing to the free radical scavenging property, the present study has been initiated to investigate the possible protective effects of AC against BLM-induced lung toxicity at an early stage of its development. To achieve this goal, some biochemical markers such superoxide dismutase and glutathione peroxidase enzymatic activities as well as reduced glutathione, malondialdehyde, nitric oxide and ATP levels were carried out in the lung tissue.

MATERIAL AND METHODS

Animals:

Adult male Sprague-Dawley rats, weighing 200-250g were obtained from the animal house of the National Cancer Institute (NCI), Cairo, Egypt. Animals were allowed free access to standard diet and water ad. libitum.

Materials:

Bleomycin hydrochloride (bleocin 15mg ampoules, from Nippon Kayaku Co., Ltd., Tokyo, Japan) was a generous gift from the NCI drug store. Acetyl-L-carnitine was kindly supplied by Dr. Menotti Calvani (Sigma-Tau Pharmaceuticals, Pomezia, Roma, Italy). All other chemicals used were of the highest analytical grade.

Experimental Design:

Bleomycin treatment protocol used in this study to develop acute lung toxicity has been previously reported **[6,18]**. A total of 40 male Sprague-Dawley rats were used and divided at random into 4 groups of 10 animals each. The first group received a daily i.p. injection of normal saline (0.5ml/200gm body weight) for 5 consecutive days and served as a control. Animals in the second group were daily i.p. injected with BLM (15mg/kg body weight) for 5 consecutive days, whereas animals in the third group were daily i.p. injected with AC (250mg/kg body weight) for 5 consecutive days. The fourth group received a daily i.p. injection of AC (250mg/kg body weight) 2 hrs before BLM (15mg/kg body weight) for 5 consecutive days. Twenty-four hrs after the last dose of the specific treatment, animals were anesthetized with ether, and blood samples were obtained by heart puncture. Serum was separated for measurement of nitric oxide. Animals were then sacrificed by decapitation after exposure to ether in a dessicator kept in a well-functioning hood and lungs were quickly excised, washed with saline, blotted with a piece of filter paper and homogenized using a Branson sonifier (250, VWR Scientific, Danbury, Conn., USA).

Determination of Reduced Glutathione, Malondialdehyde and Nitric Oxide Levels in Lung Tissue Homogenates:

GSH and MDA levels in lung tissue homogenates were determined spectrophotometrically, using the methods of Ellman **[19]** and Buege and Aust **[20]**; respectively. NO levels in serum and lung tissue homogenates were determined according to the method of Ignarro et al. **[21]**. The assay is based on the diazotization of sulfanilic acid with nitric oxide at acidic pH and subsequent coupling with N-(10 naphthyl)-ethylenediamine to yield an intensely pink colored product that is measured spectrophotometrically at 540nm.

Assessment of Enzymatic Activities:

The activity of GSHPx was determined according to the method of Lawrence and Burk **[22]**. Absorbance was measured at 340nm, and the results were expressed as umol/min/gm tissue. The changes in the absorbance at 340nm were recorded at 1-min interval for 5 min. Lung activity of SOD was assessed according to the method of Minami and Yoshikawa **[23]**. In brief, this method depends on computing the difference between auto-oxidation of pyrogallol alone and in the presence of cytosolic fraction that contains the enzyme. Enzymatic activity was expressed as ug/gm of tissue.

Determination of Adenosine Triphosphate:

ATP was determined in lung tissue using HPLC according to Botker et al. **[24]**. In brief,

lung tissue was homogenized in ice-cold 6% perchloric acid, centrifuged at 1000 rpm for 15 min at 0.5ºC, and the supernatant fluid was injected into HPLC after neutralization to pH 6-7. Chromatographic separation was performed at a flow rate of 1.2ml/min, using ODS-Hypersil, 150 x 4.6mm I.D., 5um column (Supelco SA, Gland, Switzerland) and 75mM ammonium dihydrogen phosphate as mobile phase. The peak elution was followed at 254nm.

Statistical Analysis:

Differences between obtained values (mean ±SD, n=10) were carried out by one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. A *p*-value of 0.05 or less was taken as a criterion for a statistically significant difference.

RESULTS

The effects of BLM, AC and their combination on the levels of GSH and MDA in lung tissues are shown in table (1). Administration of BLM (15mg/kg, I.P.) for 5 consecutive days resulted in a significant (340%) increase in MDA ($p<0.001$) and 39% decrease in GSH ($p<0.001$) as compared to the control group. Treatment of rats with AC for 5 days did not lead to any significant change in both GSH and MDA. Administration of AC, 2 hrs prior to BLM resulted in reversal of BLM-induced increase in MDA and decrease in GSH levels in lung tissues although these values were still significantly different than those of the controls.

Table (2) shows the effects of BLM, AC and their combination on the activity of SOD and GSHPx in lung tissues. Administration of BLM (15mg/kg, i.p.) for 5 consecutive days resulted in a significant 35% ($p<0.0001$) and 54% ($p<0.001$) decrease in the activity of SOD and GSHPx, respectively, as compared to the control group, however, AC induced non-significant change. Administration of AC to BLM-treated rats resulted in a significant (17% and 31%) increase in the activity of SOD and GSHPx ($p<0.05$ and $p<0.001$) respectively, as compared with BLM alone, and significant decrease in SOD and GSHPx, as compared to controls.

To evaluate the involvement of metabolic damage in BLM-induced lung toxicity, the effects of BLM, AC and their combination on the level of ATP in lung tissue were investigated (Table 3). Treatment of rats with BLM resulted in a significant (44%) decrease in ATP level as compared to control group ($p<0.001$). Administration of AC 2 hrs prior to BLM resulted in complete reversal of BLM-induced decrease in ATP level in lung tissue to the control values.

Table (4) shows the effects of BLM, AC and their combination on the level of NO in serum and lung tissue. Administration of BLM resulted in a significant (191%) increase in NO production in lung tissues ($p<0.001$) and non-significant change in serum NO as compared to the control group ($p>0.05$). Treatment of rats with AC for 5 days did not lead to any significant change in the level of NO in both serum and lung tissues. Administration of AC 2 hrs prior to BLM tended to decrease the increased level of NO observed in the BLM-treated group to values lesser than that of the control group.

Table (1): Effect of Bleomycin, acetyl-L-carnitine and their combination on the levels of malondialdehyde and glutathione in lung tissue.

Treatment Groups	MDA nmol/gm tissue	GSH µmol/gm tissue
Control	172.25±32.5	1.82±0.37
BLM	586.21±77.3*	0.70±0.08*
AC	150.54±30.2	2.12±0.26
AC+BLM	268.75±37.7*#	1.60±0.15*#

Data are presented as mean ±SD, n=10.
* Indicates significant change as compared with control at $p<0.05$.
# Indicates significant change as compared with BLM at $p<0.05$.

Table (2): Effect of Bleomycin, acetyl-L-carnitine and their combination on the activity of superoxide dismutase, glutathione peroxidase in lung tissue.

Treatment Groups	SOD µgm/gm tissue	GSH-Px umol/min/gm tissue
Control	76.79±11.49	16.58±1.82
BLM	49.91±2.17*	7.65±1.50*
AC	70.61±5.77	14.27±1.04
AC+BLM	62.9±3.11*#	12.80±1.16*#

Data are presented as mean ±SD, n=10.
* Indicates significant change as compared with control at $p<0.05$.
# Indicates significant change as compared with BLM at $p<0.05$.

Table (3): Effect of Bleomycin, Acetyl-L-carnitine and their combination on adenosine triphosphate content in lung tissues.

Treatment Groups	Adenosine Triphosphate nmol/gm potein
Control	26.4±6.8
BLM	14.8±4.6*
AC	30.8±8.1
AC+BLM	26.6±6.6#

Data are presented as mean ±SD, n=10.
* Indicates significant change as compared with control at $p<0.05$.
# Indicates significant change as compared with BLM at $p<0.05$.

Table (4): Effect of Bleomycin, Acetyl-L-carnitine and their combination on the level of nitric oxide in serum and lung tissue.

Treatment Groups	Nitric Oxide	
	nmol/gm tissue	µmol/ml
Control	15.004±2.63	4.01±0.78
BLM	43.65±5.23*	3.83±0.38
AC	21.12±5.84	4.33±0.61
AC+BLM	10.75±2.59*#	4.17±0.44

Data are presented as mean ±SD, n=10.
* Indicates significant change as compared with control at $p<0.05$.
# Indicates significant change as compared with BLM at $p<0.05$.

DISCUSSION

Bleomycin, a highly effective anticancer drug, is known to produce lung toxicity that limits its clinical use **[25-27].** Many experimental protocols have been reported to induce lung toxicity with BLM depending on the frequency of administration and/or the duration of the study, whether acute or chronic exposure. The protocol employed in the current study has been reported in previous studies which have demonstrated that BLM provokes early lung lesions that preceed the fibrotic changes **[6,18,28,29].**

Data from this study revealed that BLM significantly increased MDA and NO production in lung tissues. This effect could be a secondary event, following BLM-induced increase in free radical generation and/or decrease in lipid peroxidation protecting enzymes. Previous studies have reported that BLM-induced lung toxicity is related to redox cycling of an iron-BLM complex which in turn catalyzes the formation of ROS with ultimate progression of lipid peroxidation **[30,31].**

Nitric oxide is a volatile diatomic free radical that plays physiological roles in normal **[32]** as well as tumor tissues **[33].** Hopkins et al. **[34]** reported that, inflammation in the lung can lead to increased expression of inducible nitric oxide synthase (iNOS) and enhanced NO production. It has been postulated that the resultant highly reactive NO metabolites may have an important role in host defense, although they might also contribute to tissue damage. The contribution of NO in organ toxicity induced by anticancer drugs has been previously reported **[35, 36].**

In the current study, the observed increase in both reactive oxygen and nitrogen species after BLM treatment was parallel to the decrease in GSH, SOD and GSHPx. Contrary to our results, Shouman et al. **[37]** reported that chronic administration of BLM induced significant increase in SOD and GSH. This discrepancy could be attributed to the differences in the animal species, route of administration, schedule of treatment and the time intervals adapted for measurement. On the other hand, Kehrer **[38]** reported that, intratracheal administration of BLM to rats was of little effect on GSH and protein sulfhydryl content of the lung after systemic dosing with this drug. An explanation for these diverting findings is that BLM treatment may cause an acute oxidative status, which lowers temporally GSH level as reported in this study and others **[39].** However, with chronic therapy there could be up regulation of GSH level. Thus, when the induced oxidative stress depletes GSH in the mitochondria, the cells compensates this depletion by increasing the activity of the cytosolic glutamylcysteine synthetase enzyme leading to elevation of GSH level **[40,41].**

In this study, the observed decrease in GSH-Px suggests an oxidative type of injury with BLM-induced damage in lung tissue. Hasegawa et al. **[42]** reported that the decrease in GSHPx is potentially ascribable to inactivation by the increase in ROS or lipid peroxides when oxidative damage is extreme.

The carnitine system, comprehensive of carnitine, its derivatives, and proteins involved

in its transformation and transport, is indispensable for glucose and lipid metabolism in cells. Two major functions have been identified for the carnitine system: To facilitate the transport of long-chain fatty acids into mitochondria for their utilization in energy-generating processes and to facilitate the removal from mitochondria of short-chain and medium-chain fatty acids that accumulate as a result of normal and abnormal metabolism. The carnitine system appears abnormally expressed both in tumor tissue, in such a way as to greatly reduce fatty acid beta-oxidation, and in non-tumor tissue. Some anticancer drugs contribute to dysfunction of the carnitine system in non-tumor tissues, which is reversed by carnitine treatment, without affecting anticancer therapeutic efficacy **[43,35,36].**

In this study, administration of AC 2hrs before BLM significantly attenuated BLM-induced increase in both reactive oxygen and nitrogen species. These results suggest that AC has a free radical scavenging activity. Previous studies have reported that both L-and D-forms of carnitine and its short chain derivatives including AC have similar non-enzymatic free radical scavenging activity **[43,44]**. Yasui et al. **[16]** reported that AC has antioxidant activity towards oxidative stress via an inhibition of the increase in lipid hydroperoxidation observed in the brain of untreated senescence - acceleration - prone 8 mice (SAMP8). Recently, Arafa and Sayed-Ahmed **[17],** reported that the protective effects of AC and propionyl-carnitine against alcohol-induced gastric lesions was mediated through both enzymatic and non-enzymatic antioxidant mechanisms. Moreover, AC prevented BLM-induced ATP depletion in lung tissues. AC may facilitate the beta-oxidation of fatty acids in mitochondria to generate ATP, thereby, minimizing the toxic effects of free forms of long chain fatty acids in mitochondria. AC, normally produced in mitochondria, is a precursor of acetyl CoA in the tricarboxylic cycle **[46]**. Hagen et al. **[47]** reported that AC supplementation have the ability of increasing mitochondrial function and general metabolic activity without a concomitant increase in oxidative stress. In conclusion, data from this study suggests that the protective effect of AC against BLM-induced acute lung injury could be due to its antioxidant activity and preserving mitochondrial function and improving ATP production.

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