THE ROLE OF NITRIC OXIDE IN ACUTE LUNG INJURY : A HISTOLOGICAL AND BIOCHEMICAL STUDY

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INTRODUCTION

Acute lung injury (ALI) is a disease characterized by an early phase of diffuse and severe inflammatory reaction of the lung parenchyma with loss of compartmentalization followed by a late fibroproliferative phase with fever (without a source of infection) and inability to improve the lung function (Meduri et al., 1995).

It remains an important contributor to the morbidity and mortality of patients in intensive care units throughout the world (Bernard et al., 1994). The most common causes are infection, sepsis, aspiration and trauma. Since trials of anti-inflammatory therapies in ALI have shown little benefit the exact mechanism by which the lungs are injured remains controversial (Numata et al., 1998).

Recent investigations suggested that nitric oxide (NO) may play a role in acute lung injury, both beneficial and detrimental roles have been proposed (Koristof et al., 1998 and Matsuo, 1999).

Nitric oxide (NO) is a highly reactive radical synthesized from the amino acid L-arginine by the action of nitric oxide synthases (NOS) (Palmer et al., 1987). Several isoforms of NOS have been identified and divided into two categories with different regulation and activities (Moncada et al., 1991).

The constitutive NOS (c-NOS) which exits in endothelial and neuronal cells and comprises the low output path on demand in homeostatic processes such as neurotransmission or blood pressure regulation (Lowestein et al., 1994). In addition, there are inducible isoforms (i-NOS) that may be expressed after exposore to endotoxin and certain cytokines (IL-1, TNF) in macrophages, mast cells, neutrophils and endothelial cells (Gelleret et al., 1993).

Induction of i-NOS is a much greater stimulus of NO production than activation of c-NOS (**Beckman et al., 1992**). Under physiologic states, NO may serve a protective function by scavenging superoxide to protect lung tissues, but the excessive production of NO may contribute to tissue damage in which NO reacts with superoxide to form peroxynitrite, a strong oxidant. It is suggested that peroxynitrite is an important oxidant in various tissues (**Ischiropoulos et al., 1992**).

So, this study was conducted to evaluate the effects of either increased or decreased endogenous production of nitric oxide on the pathophysiological course of endotoxaemia-induced acute lung injury using histological and biochemical methods.

MATERIALS AND METHODS

An animal model of endotoxaemia-induced lung injury was prepared then either the nitric oxide substrate or the nitric oxide synthase inhibitor was used.

Drugs used :
1. Caerulein sulfated : (Sigma chemical Co.)
It is an analogue of cholecystokinin used to prepare endotoxaemia-induced lung injury.
It is white powder, dissolved in gelatin (16% w / v) to prolong its absorption. Given at dose 12 ug / kg every 8 hours for 2 days (**Frossard et al., 2002**).

2. L-arginine free base : (Sigma chemical Co.)
It is the substrate of nitric oxide synthesis i.e. nitric oxide donor. Given at dose 200 mg / kg every 8 hours for 2 days (**Andrzejewska and Jurkowska, 1999**).

3. N-G-Nitro - L-arginine (L-NNA) : (Sigma chemical Co.)
It is NOS inhibitor. Given at dose 10 mg / kg every 8 hours for 2 days (**Andrzejewska and Jurkowska, 1999**).

B) Animals :
Thirty two adult male albino rats were used. Their weights ranged between 170 - 200 grams. They were kept under hygienic conditions, fed ad libitum and allowed free water supply. Animals were divided into four equal groups. Each group was kept in a separate cage.

Group I :

Received subcutaneous saline injections in the same way as the experimental animals and served as control.

Group II :

Received caerulein subcutaneous injections for two days.

Group III :

Received subcutaneous injections of Caerulein and L-arginine for two days.

Group IV :

Received subcutaneous injections of Caerulein and L-NNA for two days.

The experiment was terminated five days after the last injection, coinciding with the end of early phase of acute lung injury **(Anderson and Thielen, 1992)** by sacrifaction of animals under chloroform anaesthesia.

II. Methods :

A) Pulmonary gas exchange :

The partial pressures of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) and pH of arterial blood were measured just before sacrifaction using a blood gas analyzer (Radio meter ABL 520 Copenhagen) **(Numata et al., 1998)**.

The mean values of all data of the studied groups were calculated and statistically compared using Duncan test **(Mould, 1989)**.

B) Histological study :

Specimens from both lungs of all groups were fixed in 10% formol saline, then processed for paraffin blocks. Sections were cut at 5 μm thickness. They were stained by HX / E and subjected for immunostaining using the polyclonal inducible nitric oxide (i-NOS) antibodies.

Immunostaining for inducible nitric oxide synthase (i-NOS) :

It was carried out by Avidin-Biotin complex (ABC). The primary antibody used was rabbit polyclonal antibody (from Lab vision NeoMarkers). Antigen retrieval required boiling of the tissue sections in 10 mm citrate buffer, for 10 minutes. The primary antibody was applied for 60 minutes. After incubation the avidin-biotin / peroxidase system was used. The bound peroxidase was visualized by incubation for

15 minutes with DAB reagent (Diamino-Benzidine). Finally sections were counter-stained with Mayer's heamatoxylin **(Bancroft and Cook, 1994).**

Negative tissue control :

The application of the primary antibody was omitted and replaced with normal mouse serum **(Bancroft and Cook, 1994).**

Positive tissue control :

Sections of human placenta known to be positive for i-NOS were processed in the same way as the studied sections **(Brancroft and Cook, 1994).**

Image analysis of i-NOS immunostaining :

The immunostained lung sections were examined by Leica Quin 500 image an-alyzer computer system (England). It was used to measure the area and area percent of positive i-NOS immunoreaction seen as cytoplasmic brown colour.

RESULTS

I. Histological results :
Group I (Control group) :

Examination of HX E - stained lung sections of control rats revealed the normal histological pattern of several, long winding passages (alveolar ducts) which opened along their length into numerous alveolar sacs and alveoli. The lung alveoli in the form of air spaces exhibited normal thickness of their walls (Fig. 1 - a). They were lined with type (I) and type (II) pneumocytes (Fig. 1 - b).

The bronchioles showed normal simple columnar epithelial lining (Fig. 1 - c). The adjacent arteries showed normal thickness of their walls and were surrounded by normal alveoli.

Group II :

Six rats of this group survived till termination of the experiment. Examination of HX / E-stained lung sections revealed diffuse inflammatory reaction of lung pa-renchyma (Fig. 2 - a). Marked inflammatory cell infiltrate was present in the inter-stitium leading to interstitial thickening. The infiltrate composed predominantly of polymorphnuclear leucocytes with eosinophils. Prominent type (II) Pneumocytes could be seen (Figs. 2 - b, c).

Pink-staining exudate and vascular congestion were observed with extravasations of red blood corpuscles (Fig. 3 - a).

The bronchioles revealed pseudostratification of their epithelial lining. Their lumina contained fibrin strands and shedded cells as well as Polymorphs (Fig. 3 - b).

Group III :

Only four rats of this group survived for five days after caerulein and L-arginine injections. Worsening of the histological picture of lung injury was seen in HX / E-stained sections (Fig. 4 - a). More profuse mononuclear cell infiltrate was present in alveolar walls with markedly thickened alveolar septa. It consisted mainly of clusters of polymorphs and esoinophils. Flooding of alveoli with serofibrinous exudates was evident. Prominence of type (II) Pneumocytes was noticed. The blood vessels were severely congested with extravasations of RBCs showing roleaux appearance (Fig. 4 - b).

The bronchioles were lined with hyperplastic epithelium, their lumina were filled with inflammatory cells, shedded cells and fibrin strands, that led to complete obliteration of some lumina (Figs. 5 - a, b). Peri-bronchiolar aggregates consisting of lymphocytes were also seen (Fig. 5 - c).

Group IV :

All rats of this group survived till time of sacrifice.

Compared with the previous groups, the histological picture of lung sections revealed normal thickness of most alveolar walls with little fibrinous exudate (Fig. 6 - a). Minimal mononuclear cellular infiltrate was present in the interstitium. Slight congestion of pulmonary capillaries was notable (Fig. 6 - b).

The bronchioles showed almost normal epithelial lining with scanty cell infiltrate and reduced peri-bronchiolar lymphocytic aggregation (Fig. 6 - c).

II. Immunohistochemical results :

All results were compared with the negative tissue control sections.

Sections from the control group revealed absence of any positive immunostaining with the polyclonal antibody to i-NOS (Fig. 7). Lung sections of group (II) showed positive cytoplasmic immunoreaction within the alveolar epithelium predominantly in pneumocytes type (II). Patchy staining of inflammatory cells was also observed (Figs. 8 - a, b). In group (III), which received L-arginine together with caerulein, positive immuno-staining was seen throughout the lung sections. The

interstitium, alveolar epithelium (mainly pneumocytes type II) and bronchiolar exudates were strongly stained (Figs. 9 - a, b, c). However, in group (IV) treated with L-NNA, iNOS immunostaining was markedly attenuated and only weak staining of alveolar walls was seen (Fig. 10).

The area percent of i-NOS immunoreaction in the studied groups is shown in Fig. (11).

III Pulmonary gas exchange :

Analysis of arterial blood gases revealed that, the mean PaO2 in the control group (Group I) was 104.44 ± 1.02 mmHg. Significant deterioration of PaO2 was found in group (II) which received caerulein injections only and group (III) where L-arginine was co-administered with caerulein. It reached 73.55 ± 3.07 mmHg and 58.52 ± 1.01 mmHg respectively.

In contrast, treatment with L-NNA (NOS inhibitor), in group (IV) resulted in an increase of PaO2 to 94.31 ± 2.92 mmHg which was non significant compared to the control.

The mean PaO2 in the studied groups are shown in Fig. (12) and Table (1).

In the control group, the mean PaCO2 was found to be 40.45 ± 0.69 mmHg and mean pH was 7.35 ± 0.11. Compared with these values, in groups (II) and (III) there were significant increase in PaCO2 to 48.63 ± 0.68 mmHg and 54.31 ± 0.65 mmHg respectively. This was also accompanied with significant decrease in pH to 7.12 ± 0.19 in group (II) and to 6.98 ± 0.19 in group (III). Non significant increase of PaCO2 or decrease of pH was noticed in group (IV).

The mean values of PaO2, PaCO2 and pH in the studied groups are represented in Table (1).

Table 1 : The mean $\pm$ SD of PaO_2, $PaCO_2$ and pH in the studied groups.

Group	PaO_2	$PaCO_2$	pH
Group I (control)	104.44 ± 1.02	40.46 ± 0.69	7.35 ± 0.11
Group II	$73.55 \pm 3.07*$	$48.63 \pm 0.68*$	$7.12 \pm 0.19*$
Group III	$58.52 \pm 1.01*$	$54.31 \pm 0.65*$	$6.98 \pm 0.19*$
Group IV	94.31 ± 1.03	42.11 ± 0.75	7.26 ± 0.06

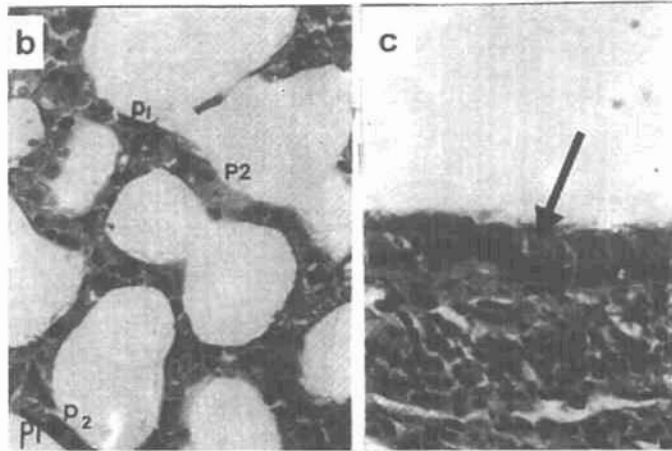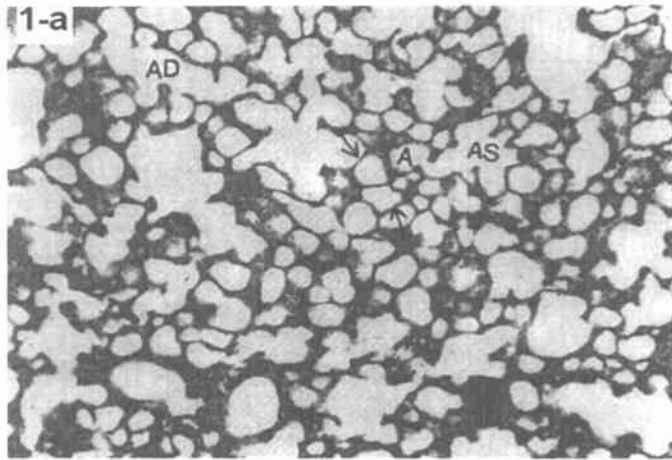*Significant difference compared to control (P < 0.05).

Fig. (1) :Photomicrographs of sections in lungs of control rats
showing :
a) Alveolar ducts (AD) with numerous alveolar sacs (AS) and
alveoli (A). Normal alveolar septa (arrows) between adjacent
alveoli.

(Hx. & E.; x 100)
b) Epithelial lining of alveoli consisting of flattened type I
(p1) and rounded type II (p2) pneumocytes.

(Hx. & E.; x 400)
c) Simple columnar epithelial lining of bronchioles.
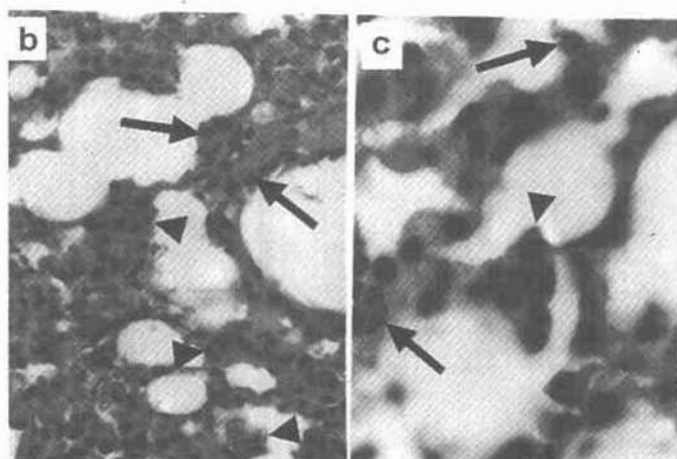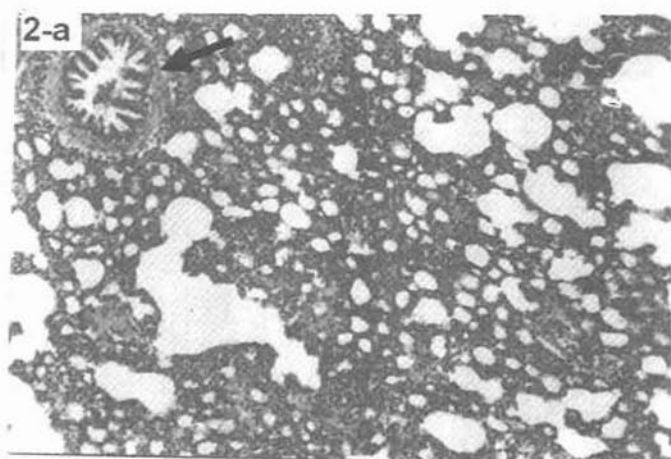
(Hx. & E.; x 400)

Fig. (2) :Photomicrographs of sections in lungs of caerulein-
injected rats (group II) showing :
a) Diffuse inflammatory reaction of lung parenchyma. A
bronchiole with intra-luminal infiltrate could be seen (arrow).
(Hx. & E.; x 100)
b) Higher magnification showing thickened alveolar septa
with cellular infiltrate (arrows) and prominent type II pneu-
mocytes (arrow heads).
(Hx. & E.; x 400)
c) Polymorphnuclear leucocytes (arrows) and an eosinophil
seen in the inter-alveolar septa (arrow head).
(Hx. & E.; x 1000)
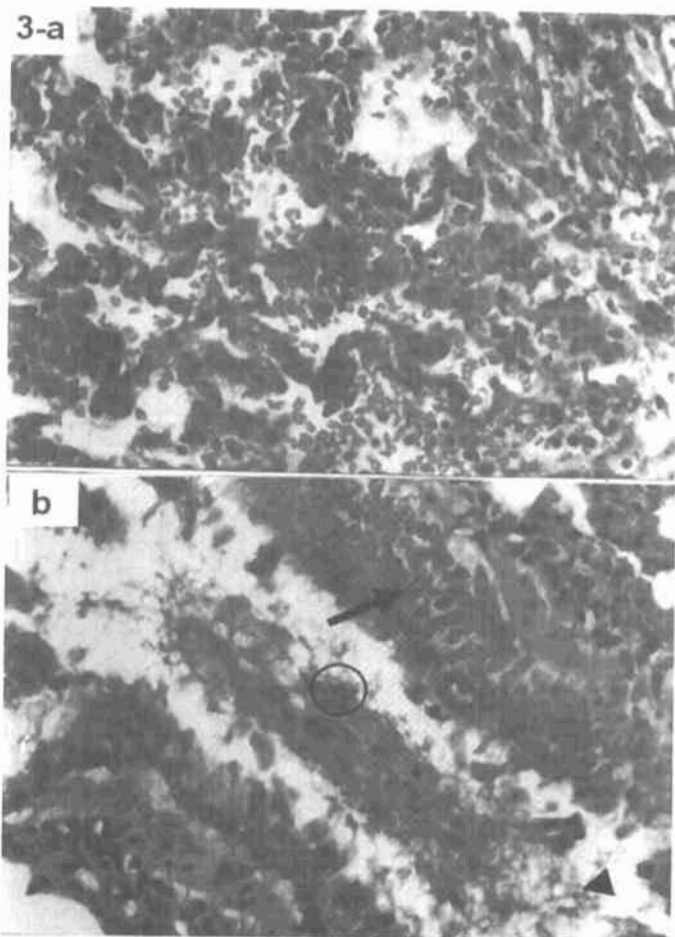
Fig. (3) :Photomicrographs of sections in lungs of caerulein
injected rats (group II) showing :
a) Marked congestion and extravasations of many RBCs.
                                        (Hx. & E.; x 400)
b) Pseudostratification of bronchiolar epithelium (arrow) with
intra-luminal cell infiltrate of neutrophils (circled) and fibrin
(arrow heads).
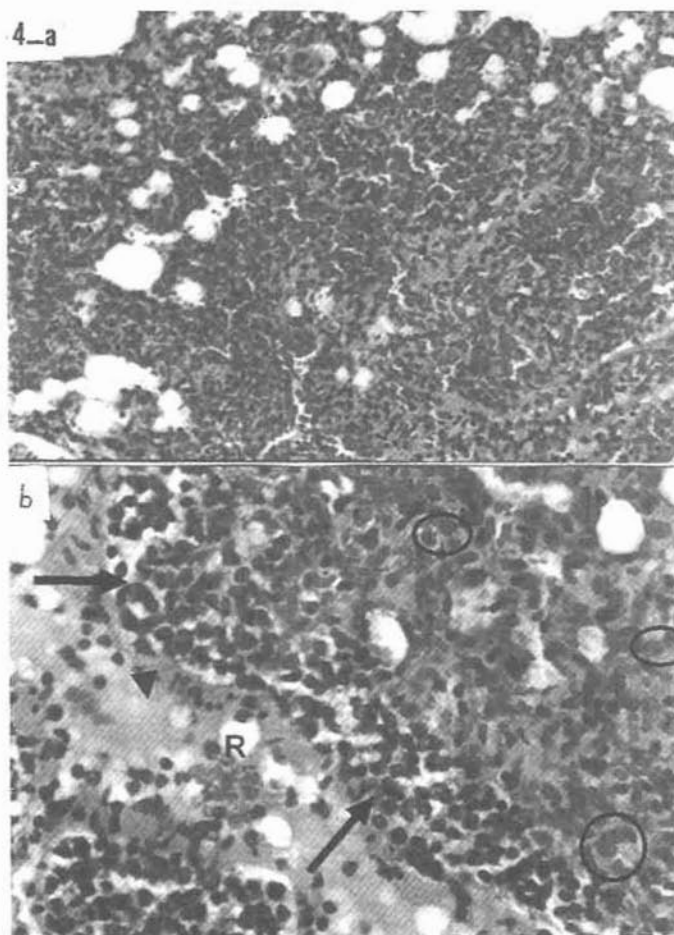
                                        (Hx. & E.; x 400)

Fig. (4) :Photomicrographs of sections in lungs of group III
(caerulein + L-arginine) showing :
a) Loss of normal lung architecture and massive pulmonary
hemorrhage.

(Hx. & E.; x 200)

b) Marked mononuclear inflammatory cellular infiltrate
(arrows) and Fibrinous exudate could be noted (arrow head).
Prominent type II pneumocytes were seen (circled) as well as
roleaux appearance of RBCs (R).
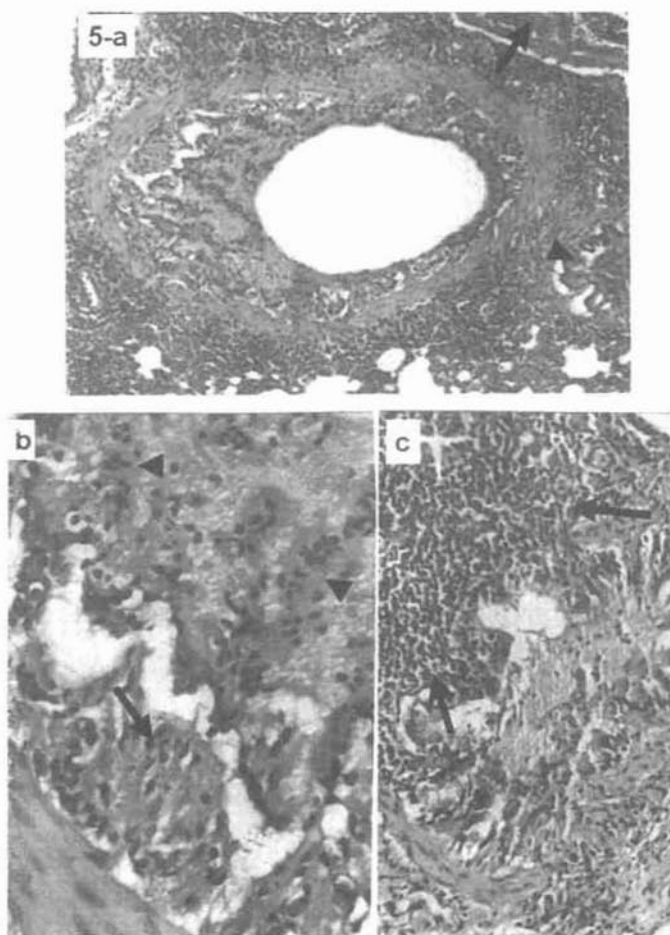
(Hx. & E.; x 400)

Fig. (5) :Photomicrographs of lung sections of group III
(receiving caerulein + L-arginine) showing :
a) A bronchiole with intra-luminal infiltrate and fibrin strands
Part of a smaller bronchiole with nearly obliterated lumen
(arrow head) and congested blood vessel (arrow).

(Hx. & E.; x 100)
b) Higher magnification of a part of the bronchiole revealing
hyperplastic epithelial lining (arrow), shedded epithelial cells,
and fibrin threads (arrwo heads).

(Hx. & E.; x 400)
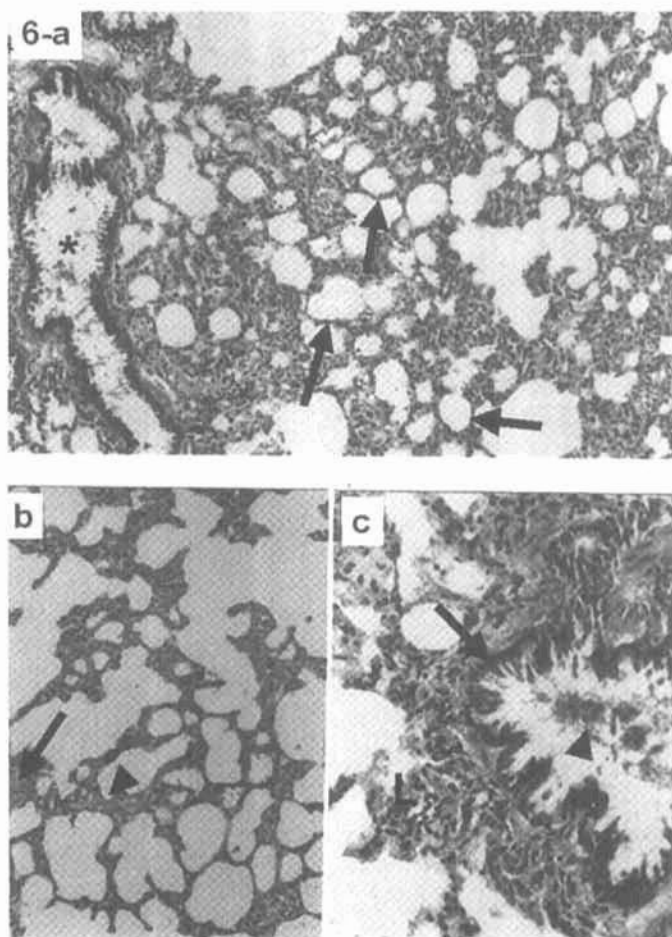c) Peri-bronchiolar lymphocytic aggregation (arrows).
(Hx. & E.; x 200)

Fig. (6) :Photomicrographs of lung sections of group IV
(receiving caerulein + L-NNA injections) showing :
a) Alveolar septa of nearly normal thickness (arrows). A bron-
chiole with its simple columnar epithelial lining could be seen
(*).

(Hx. & E.; x 100)
b) Mild congestion of pulmonary capillaries. (arrows).

(Hx. & E.; x 100)
c) A bronchiole with reduced both peri-bronchiolar lympho-
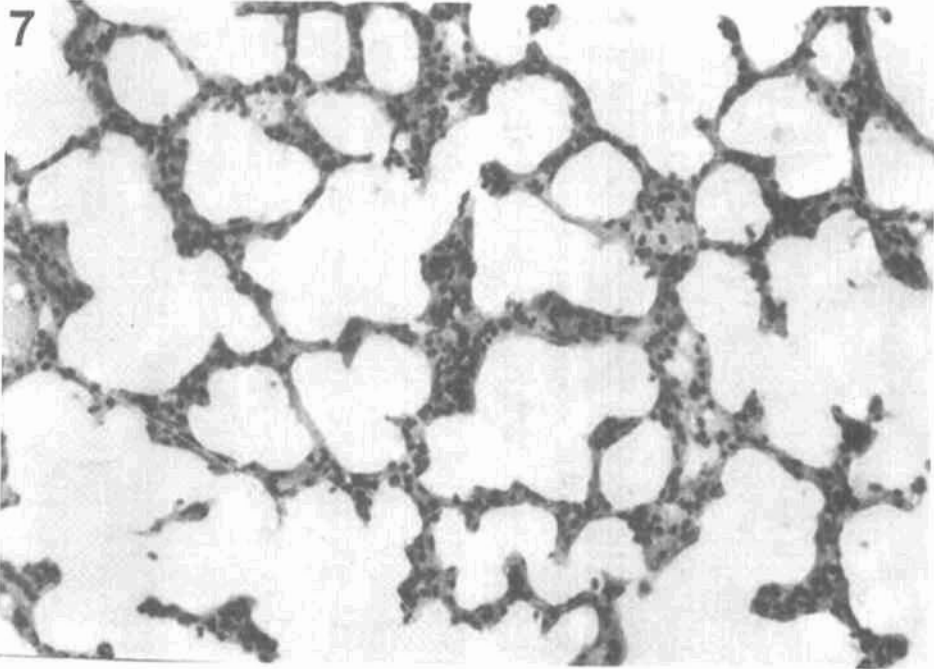cytic aggregation and intra-luminal infiltrate.

(Hx. & E.; x 200)

Fig. (7) : A Photomicrograph of lung sections of control rat immunostained with i-NOS antibody revealing negative immunostaining.
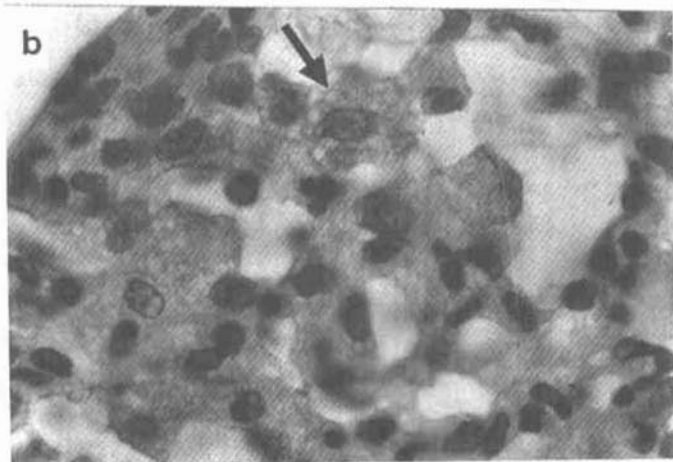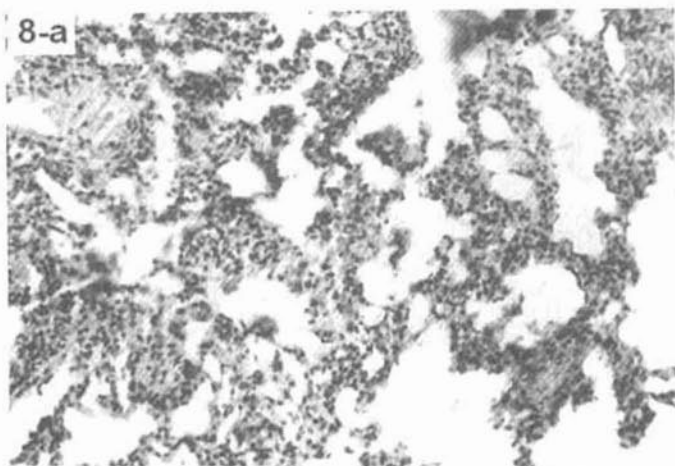
(x 200)

Fig. (8) :Photomicrographs of lung sections of group II im-
munostained with i-NOS antibody showing :
a) Positive immunoreaction within alveolar epithelium as well
as some inflammatory cells.

(x 200)
b) Cytoplasmic immunoreactivity within pneumocytes type II
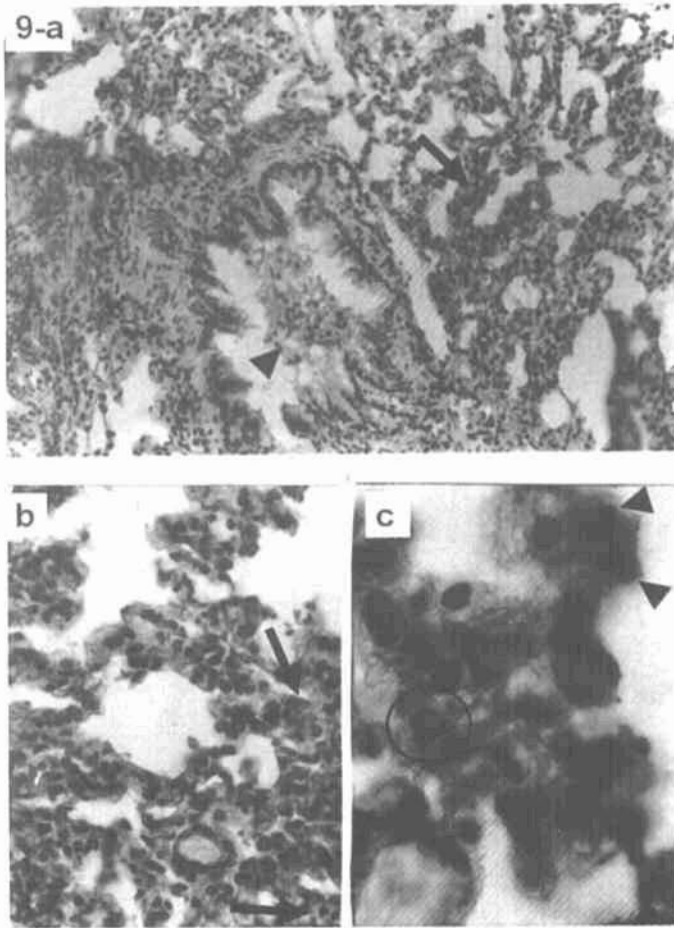(arrow).

(x 1000)

Fig. (9) :Photomicrographs of i-NOS immunostained lung
sections of group III showing :
a) Widespread positive reaction throughout lung section, the
interstitium (arrows) and intra-bronchiolar infiltrate (arrow
heads).

(x 100)
b) Positive i-NOS immune reaction within pneumocytes type
II (arrows).

(x 400)
c) Positive cytoplasmic staining within Pneumocytes type II
(arrow heads) and an eosinophil (circled).
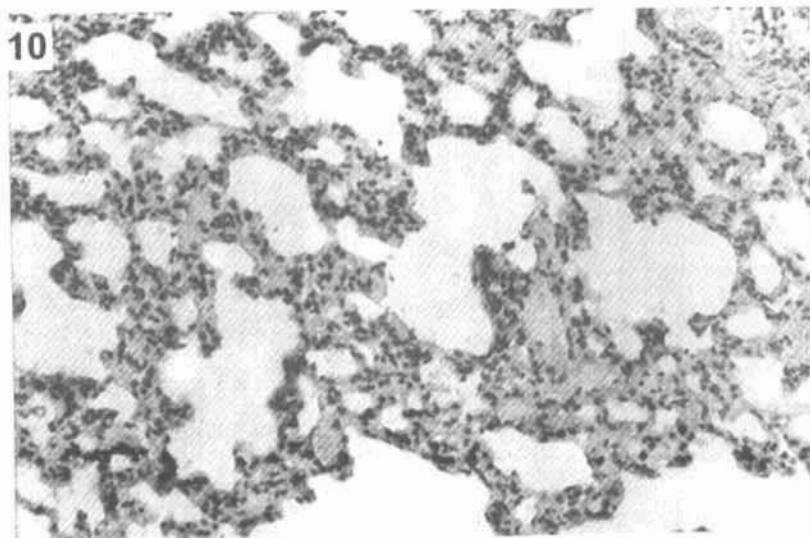
(x 1000)

Fig. (10) :A photomicrograph of i-NOS immunostained lung section of group IV showing very weak staining within alveolar walls.
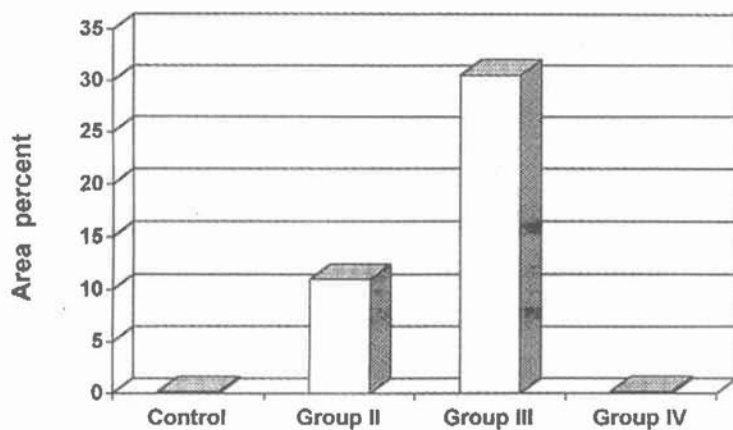
(x 200)

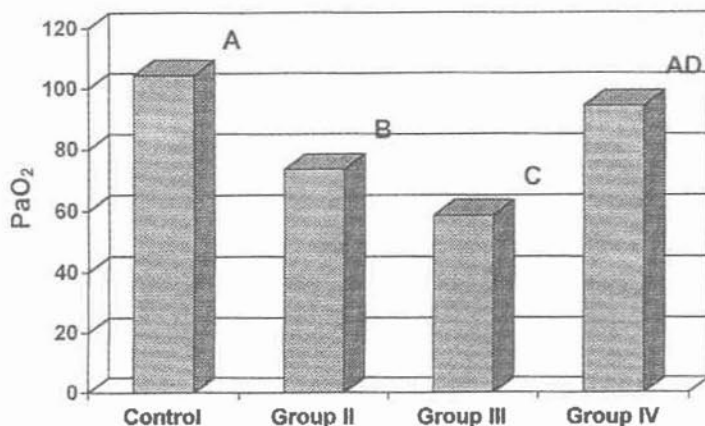**Fig. (11)** :Mean area % of i-NOS immunoreaction in the studied groups.

Fig. (12) :Mean PaO$_2$ in the studied groups.
Groups not sharing a letter are significantly different from each other (P < 0.05)

DISCUSSION

In the present work an experimental model of endotoxaemia-induced lung injury was prepared by subcutaneous injections of caerulein. The administration of caerulein results in pancreatitis associated with a significant lung injury through the release of mediators into the blood that induce within the lung the chronological expression of macrophage inflammatory peptide-2, tumor necrosis factor-alpha and intercellular adhesion molecule-1 (Frossard et al., 2002).

In human medicine, this stage of lung disease is frequently referred to as adult respiratory distress syndrome (ARDS) which is characterized by acute onset of diffuse and severe inflammatory reaction of lung parenchyma resulting in protein rich exudative oedema. These histopathological changes are accompanied by arterial hypoxaemia, abrupt increase in the PaCo2 (may be decreased in the early stage of ARDS due to hyperventilation) and a corresponding decrease in pH (Meduri et al., 1991).

It had been shown that caerulein injections in group (II) resulted in diffuse inflammatory reaction of lung parenchyma with sequestration of leucocytes mainly polymorphs and thickening of alveolar walls, associated with hypoxaemia (PaO2 73.55 ± 3.07 mmHg) and acidosis. Co-administration of L-arginine (NO precursor) led to worsening of the lung architecture with more severe hypoxaemia (PaO2

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58.52 ± 1.01 mmHg) and acidosis. On the other hand, treatment with L-NNA (NO inhibitor) attenuated these changes to a great extent and resulted in increased PaO2 to 94.31 ± 2.92 mmHg.

It was reported by **Numata et al. (1998)** that in sepsis, toxic products activate systemic host defenses including, neutrophils, monocytes, macrophages and endothelial cells that produce toxic host mediators such as cytokines, kinins and Nitric oxide (NO). They also stated that neutrophils have been implicated specifically in the pathogenesis of most cases of human sepsis. Consisted with this report, in the present study neutrophils accumulated in the interstitium of lung with induction of sepsis and treatment with NOS inhibitor attenuated the neutrophil sequestration.

This also goes with preliminary data from ongoing clinical trials which indicate that inhibitors of NOS activity exert beneficial effects in septic shock **(Thiemermann, 1997)**. Also, in several animal models of inflammation, a selective inhibitor of i-NOS, N-iminoethyl L-lysine suppressed the infiltration of inflammatory cells **(Wolkow, 1998)**.

It was found that exogenous nitric oxide elicits chemotaxis of neutrophils **(Numata et al., 1998)**. Therefore, attenuation of neutrophil infiltration may be explained by inhibition of NO production. **Beckman et al. (1992)** reported that large amounts of NO Produced by i-NOS induction interacts with oxygen free radicals derived from neutrophils and macrophages to form peroxynitrite which is a potent and versatile oxidant that can attack many types of biologic molecules.

On the other hand, **Liu et al. (2001)** concluded that inhibition of NO synthesis in a rat model of acute lung injury induced by hepatic ischemia-reperfusion, not only augments ischemic liver injury but also enhances the systemic inflammatory response and exacerbates remote lung injury. This may be attributed to the different mechanisms of inducing lung injury.

The inflammatory reaction in the lung was associated with pulmonary congestion with consequent presence of RBCs, this may be explained by the fact that nitric oxide stimulates soluble guanyl / Cyclase resulting in increased level of cyclic GMP in lung smooth muscle cells. The gating of K^+ and Ca^2 channels by cyclic GMP binding is thought to play a role in nitric oxide mediated vasodilatation **(Weinberger et al., 1999)**. Also according to **Motamen et al. (1998)**. Nitric oxide's function in vasomotor control, inflammation and signal trans-duction makes it an attractive potential mediator of the capillary leak seen in acute lung injury.

The immunohistochemical study of i-NOS was done to investigate the possible source of NO generation during endotoxaemia. In caerulein injected group the

alveolar epithelium especially pneumocyts type (II) showed positive reaction. This goes with a previous study done by **Toga et al. (2001)** they isolated i-NOS and its mRNA as well nuclear factor kappa B (NF, kappa B) from type II cells during lung inflammation. They reported that effects of corticosteroids may be in part through inhibition of both i-NOS and NF, kappa B activation. Treatment with L-arginine, resulted in intense i-NOS immunostaining seen in alveolar epithelium and inflammatory cells. According to **Weinberger et al. (1999)** nitric oxide is produced by many cell types in the lung. On the contrary **Wang et al. (2002)** suggested that, the microvascular protein leak in sepsis induced acute lung injury is uniquely dependent on i-NOS in inflammatory cells with no obvious contribution of i-NOS in pulmonary parenchymal cells. It had been found that mice deficient in i-NOS gene were more resistant to endotoxaemia induced lung injury than wild-type mice as reported by **Koristof et al. (1998)**.

The observations of this study suggest that increased intra-pulmonary generation of nitric oxide has an important role in the pathogenesis of endotoxaemia induced-acute lung injury. Pneumocytes type (II) and some inflammatory cells (Polymorphs) are possible sources of endogenous NO production. Accordingly, Nitric oxide synthase inhibitors may be effective in reversing the lung injury.

SUMMARY

Respiratory complications are major factors contributing to death in acute toxaemia. They are characterized by complex interactions among cytokines, chemokines, adhesion molecules and leucocytes. Pro-inflammatory cytokines have been implicated in the up regulation of the inducible form of nitric oxide synthase (i-NOS) which produces large amounts of nitric oxide (NO).

This study was conducted to investigate the effects of increased or decreased endogenous production of nitric oxide on the course of acute lung injury.

Thirty two adult male ambino rats were used. Divided into four equal groups. Group (I) received saline injections and served as control. Acute lung injury was induced in the other three groups by subcutaneous injections of caerulein. Group (II) was left untreated. In group (III) L-arginine (NO precursor) was co-administrated with caerulein, while group IV was treated simultaneously with L-NNA (NOS inhibitor). Results were evaluated using histological and biochemical methods. The histological picture of lung injury was associated with hypoxemia. These changes worsened with the co-administration of L-arginine. Treatment with L-NNA greatly

improved the condition, as manifested by the microscopic examination of lung sections and through analysis of blood gases. Immunohistochemical study of inducible nitric oxide synthase revealed that pneumocytes type (II) and some inflammatory cells (polymorphs) may be the possible source of endogenous NO production in acute lung injury.

It could be concluded that increased intra-pulmonary generation of nitric oxide by the action of inducible nitric oxide synthase plays a critical role in the pathogenesis of acute lung injury. So it is suggested that i-NOS inhibitors may have a potential in the treatment of acute respiratory distress syndrome.

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الملخص العربى

دور أكسيد النيتريك فى الإصابة الحادة للرئة : دراسة هستولوجية وبيوكيميائية

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تعتبر المضاعفات التنفسية من أهم الأسباب المؤدية للوفاة فى حالات سمية الدم الحاده والتى تتميز بتفاعلات معقدة بين السيتوكينات والكيموكينات والجزئيات اللاصقة وكرات الدم البيضاء . هذا وتلعب السيتوكينات فى مراحل الالتهاب المبكره دوراً فى تنظيم عمل الإنزيم المحفز على تكوين اكسيد النتريك .

أجريت هذه الدراسة لفحص تأثير زيادة أو نقص انتاج اكسيد النتريك داخلياً على تطور الإصابة الحادة للرئة .

قسمت ذكور الفئران البيضاء إلى أربعة مجموعات متساوية (٨ فئران) استخدمت الأولى كمجموعة ضابطه تم حقنها بمحلول ملحى . فى حين أحدثت اصابة الرئة الحادة فى المجموعات الثلاث الأخرى عن طريق الحقن تحت الجلد بمادة الـ Caerulein ، وتركت المجموعة الثانية كما هى فى حين اعطيت المجموعة الثالثة المادة الأولية لتكوين اكسيد النتريك L-arginine أما المجموعة الرابعة فعولجت بالمادة المثبطة للإنزيم المكون لاكسيد النتريك L-NNA ، وتم تقييم آثار هذه المعاملات هستولوجيا وبيوكيميائياً .

ظهرت إصابة الرئة الحادة فى المجموعة الثانية مصحوبة بنقص الضغط الجزئى للاكسجين وزيادة ثانى أكسيد الكربون فى الدم ، فى حين أدت المعاملة

بالـ L-arginine فى المجموعة الثالثة إلى مضاعفة هذه الأعراض . أظهر الفحص الميكروسكوبى وتحليل الضغط الجزئى للغازات فى الدم تحسنًا ملحوظًا فى المجموعة الرابعة والتى اعطيت L-NNA .

وقد استدل من الدراسة الهستوكيميائية المناعية والتى اجريت لتحديد أماكن الإنزيم المحفز على تكوين اكسيد النتريك إلى أن الخلايا الرئوية (النوع الثانى) وبعض الخلايا الإلتهابية قد تكون مصدرًا لتكوين اكسيد النتريك فى حالات الاصابة الحادة للرئة .

وتشير نتائج هذا البحث إلى أن الزيادة الداخلية لإفراز اكسيد النتريك فى الرئة قد تلعب دورًا هامًا فى التطور المرضى للإصابة الحادة بالرئة ، وعليه فإن إستخدام مثبطات الإنزيم المكون لأكسيد النتريك قد تساهم فى علاج الأعراض الناجمة عن متلازمة مصاعب التنفس الحاده .