



EFFECTS OF PHARMACOLOGIC INTERVENTION ON OXYGENATION, LUNG WATER
AND PROTEIN LEAK IN THE PSEUDOMONAS ARDS PORCINE MODEL.

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) A porcine model of Pseudomonas induced acute lung injury was studied. It was demonstrated that circulating neutrophils are primed to produce short and long lived reactive oxygen species at a greater and faster rate after 5 hours of sepsis than at baseline. These findings were supported by complimentary in vitro studies. Static and dynamic lung compliance was significantly reduced in septic animals compared with controls. These changes were significantly attenuated by pretreatment with ibuprofen as was the priming of the neutrophil respiratory burst.					
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SUMMARY

The adult respiratory distress syndrome is a condition which occurs as a result of both direct and indirect pulmonary injury. The mortality rate for the syndrome which may affect previously fit patients is over 50% and higher where sepsis predominates. This mortality, despite modern techniques in intensive care, has hardly changed in 20 years. The pathophysiological changes in the condition result in respiratory failure requiring endotracheal intubation and mechanical ventilation, the appearance of diffuse fluffy infiltrates on chest roentgenogram in the presence of a normal pulmonary wedge pressure, an arterial:alveolar pO_2 ratio ≤ 0.2 , and a total static lung compliance of ≤ 50 ml H_2O .

At the cellular level, the lung injury is due to damage of the alveolar-capillary membrane by various mediators produced from circulating elements of the blood, most notably neutrophils. Neutrophils become "activated" by contact with a soluble or phagocytic stimulus, they break down and release many inflammatory mediators. Among the mediators produced, following a septic stimulus are the products of the cyclooxygenase and lipoxygenase systems of arachidonic acid metabolism such as the prostaglandins, leukotrienes, the complement factors C5a, C3a which are also mobilized by septic stimuli. Oxygen free radicals and tumor necrosis factor are other inflammatory mediators released by host cells.

All of these substances can be toxic to the alveolar capillary membrane and eventually may cause its disintegration with

concomitant protein leak across the damaged membrane into the lung. When the lymphatic clearance capacity of the lung is exceeded, pulmonary edema occurs and the clinical picture seen in ARDS unfolds. Pseudomonas-induced ARDS in the porcine model has been used as an effective and reproducible model of sepsis-induced ARDS in this laboratory.

Because ARDS is mediated by numerous inflammatory mediators, it is likely that treatment will require several pharmacological blocking agents. We have previously established that treatment with cimetidine, or ranitidine in combination with diphenhydramine, H₂ and H₁ blockers, respectively, and ibuprofen, a prostaglandin antagonist (CID), given i.v. at 20 and 120 minutes after pseudomonas infusion, significantly attenuates both the early hypertensive and late permeability phases of the syndrome as measured by hemodynamic parameters, blood gases, bronchoalveolar lavage protein content and extravascular lung water.

We have established that platelet-activating factor is present in pig lung after sepsis. An anti-platelet activating factor, SRI 63-675, has been shown previously to attenuate the early phase of pulmonary hypertension and possibly improve the late permeability changes in the model but caused severe hemolysis, thus making it impractical for clinical use to date. Experiments using superoxide radical scavengers in the model have not been shown to be effective in improving the response to injury in any way despite the wealth of evidence that primary oxygen radicals effect alveolar-capillary membrane damage. However the characterization of superoxide

production from neutrophils in this model has been much more clearly defined in this laboratory in the last year. Similarly the production of a group of longer lived but more potent oxidants, hypochlorous acid and monochloramines has been identified and quantified from onset to advanced sepsis. Treatment with standard cyclooxygenase blockers, in this case ibuprofen, has been shown to markedly attenuate the release of this wide variety of neutrophil oxidants. These data indicate that cyclooxygenase products of arachidonic acid are intimately involved in the respiratory burst of phagocytes. However, preliminary data would suggest that treatment of the septic ARDS animals with combination therapy CID as described above results in outpouring of large quantities of oxidants in advanced sepsis as previously demonstrated in septic animals alone. This apparent paradox is most likely explained by the fact that histamine as has been shown in-vitro, is a blocker of the respiratory burst of activated phagocytes. Thus, by adding the two anti-histamines to the treatment regime, this inhibition is removed allowing full oxidant generation. The paradoxical fact is that deterioration in hemodynamic and permeability parameters is very much attenuated with this treatment despite increased superoxide generation.

Measurement of conjugated dienes in serum as a reflection of oxygen radical activity on lipid membranes in the pseudomonas injured animals was not an accurate marker of the effects of radical scavengers.

As mentioned in a previous report, kinetic and quantitative studies of superoxide production from neutrophils separated from the pre- and post-injury phases in the model indicate that *Pseudomonas* primes these cells to produce superoxide anion at a much higher rate post-injury than pre-injury, thus implicating neutrophil generated superoxide anion in the endothelial cell damage. Alveolar macrophages retrieved from bronchoalveolar lavage in the post-injured phase are capable of generating large quantities of superoxide anion and hydrogen peroxide and may have a role in the injury process, in particular with epithelial cell damage.

Tumor necrosis factor (TNF) generated by mononuclear cells is increased in the injured animal. Following a bacterial insult there is an immediate and dramatic rise in TNF levels. This substance has been implicated in many inflammatory processes and has been seen by others to increase in humans exposed to endotoxin. Raised levels of TNF are temporally related to alterations in hemodynamic parameters in this model system.

Static and dynamic compliance in the *Pseudomonas* treated animals decreases significantly following onset of the bacterial infusion and remains decreased throughout the study period. In both ibuprofen treated animals and in CID treated animals compliance measurements were maintained at or about control levels throughout the study period. This parameter is used as an important tool in the assessment of therapeutic manipulations in the model.

FOREWORD

In conducting the research described in this report, the investigators adhere to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH 78-23, Rev. 1978).

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PROBLEM

The adult respiratory distress syndrome (ARDS), as first described by Ashbaugh (1) 20 years ago, is a pathophysiological pulmonary condition of multiple etiologies. The syndrome may be initiated by direct pulmonary injury or may be seen as the lungs' response to a remote or systemic insult. In civilian life, the most common causes of ARDS are multiple trauma, aspiration of gastric contents, sepsis and pancreatitis. In combat soldiers, the condition known as the traumatic wet lung syndrome during the Korean conflict and Da-Nang lung in the Vietnam War, is now recognized as ARDS and results from blast injuries, direct lung contusion, burn inhalation, inhalation of toxic substances, aspiration, multiple transfusions and as a complication of sepsis. As such, the condition often affects previously fit and healthy patients with a considerable mortality.

Clinically, patients are considered to have the syndrome when certain criteria are met: respiratory failure requiring endotracheal intubation and mechanical ventilation, the appearance of diffuse pulmonary infiltrates on chest roentgenogram, an initial pulmonary wedge pressure of 18 mmHg or less, an arterial to alveolar P_{O_2} ratio ≤ 0.2 and a total static lung compliance of ≤ 50 ml H_2O which increases the work of respiration with stiffer lungs and decreases oxygenation.

Applying these criteria, between 150,000 and 200,000 patients in the United States are affected annually by this syndrome. The mortality rate remains at 50% or higher where sepsis predominates (2). The mainstay of treatment is supportive therapy, treatment of the underlying disorder, the maintenance of adequate oxygenation with mechanical ventilation and positive endexpiratory pressure (PEEP), fluid balance, nutrition and antibiotics where indicated, since patients with ARDS are more susceptible to nosocomial infections.

BACKGROUND

Despite the multiple causes of ARDS, the end result is the same, i.e. damage to the alveolar-capillary membrane with increased permeability and accumulation of water and protein rich fluid in the pulmonary interstitium. Alveolar flooding occurs when interstitial and lymphatic clearance capacities are exceeded leading to decreased pulmonary compliance. The perfusion of unventilated alveoli manifested as hypoxemia is also a feature. This hypoxemia is refractory to increased inspired oxygen concentrations.

It is likely that successful treatment of ARDS will involve intervention to prevent capillary endothelial damage and protein leak across the membrane, as well as efforts to increase compliance and improve oxygenation.

Measurement of extravascular lung water by the indicator dilution technique has been used in all the animal experiments in this laboratory where such determinations were necessary for the assessment of therapeutic intervention in experimental ARDS. In addition to these methods of determining lung leak, we have added the technique of bronchoalveolar lavage to further study cell traffic across the damaged alveolar-capillary membrane and to recover inflammatory and resident cells from the alveolus in order to study their role in the development of the syndrome.

In addition to the comprehensive evaluation of clinical parameters in this model, we have also focused on a wide array of cellular, molecular and functional assessments of abnormalities in

sepsis-induced ARDS. These new parameters include assay of short and long lived oxidant generation from neutrophils and tumor necrosis factor and interleukin-1 generation from mononuclear cells. Myeloperoxidase production and gene expression in alveolar macrophages and circulating neutrophils are a new focus and neutrophil adherence receptor studies utilizing flow cytometry and a porcine pulmonary endothelial cultured cell line are another of our current interests. Assay of intracellular protein kinase C activity in phagocytes and investigation of porcine pulmonary intravascular macrophages also provide exciting new directions.

RATIONALE

Role of inflammatory mediators in ARDS.

It is clear that the lung injury in ARDS is mediated by a large number of substances and that some of these substances are inter-related and share a common final pathway or common enzyme systems. What is not clear is the exact inter-relations between these mediators. It is likely that therapeutic intervention with a specific, or a combination of specific agents, will attenuate the lungs' response to injury and thereby minimize its consequences.

Central to the lungs' response to injury in endotoxin induced ARDS are the neutrophils (3-5). Sequestration of neutrophils takes place soon after endotoxin infusion in-vivo. The exact method of neutrophil aggregation is not known, but it is hypothesized that substances such as complement C3a and C5a, leukotrienes and various other chemotactic substances are involved in the initiation of the process (6). In-vitro, plasma activated with zymosan causes neutrophil aggregation. Neutrophils become "activated" when they come in contact with a soluble or phagocytic stimulus and manifest this activation as an increased respiratory burst with an increase in oxygen consumption, activation of the hexose monophosphate shunt and generation of reactive oxygen species and their metabolic products. These products may be injurious to endothelial cells as well as deactivating enzymes and causing mutagenesis by their action on DNA.

As well as oxygen free radicals, the neutrophils, platelets, monocytes and lymphocytes can release a number of other factors which have an affect on pulmonary hemodynamics and directly on the endothelium. The products of arachidonic acid metabolism produced by the circulating elements in the blood are thought to cause the acute pulmonary hypertension seen immediately after endotoxin infusion as well as increased lung lymph flow (7-9). Products of the cyclooxygenase system of arachidonic acid metabolism are thought to cause these effects since increased plasma levels of TxB_2 and 6 Keto $\text{PGF}_{1\alpha}$ are temporally related to the initial pulmonary hypertension and increased lung lymph flow. It has been shown by previous experiments in this laboratory that these effects can be prevented by a combination of an anti-prostaglandin in conjunction with histamine receptor blockers (10). The macrophage has been implicated in the lung injury by its release of substances such as the interleukins and tumor necrosis factor (cachectin) (11,12).

The second phase of the lungs' response to endotoxin is characterized by a sustained but lower elevation in pulmonary artery pressure and an increased protein rich lymph flow secondary to an increased capillary permeability. This late phase of pulmonary hypertension and protein leak is not thought to be due to the metabolites from the arachidonic cascade; however, it is possible that oxygen-free radicals generated by neutrophils (13) are responsible for a large part of the vascular endothelial cell

damage as well as proteolytic enzymes released by degranulating neutrophils (14) all of which result in increased permeability.

Oxygen-free radicals produced by neutrophils are normally converted to non-injurious substances by the enzymes superoxide dismutase and catalase. However, when these mechanisms are overwhelmed by massive oxygen radical production ($O_2^{\cdot-}$, H_2O_2 , $OH^{\cdot}$) endothelial cell damage can occur. The reaction of hydrogen peroxide with chloride ions in the presence of the neutrophil degranulation enzyme myeloperoxidase leads to the production of hypochlorous acid which is thought by some investigators to be the most potent of the oxidants produced by neutrophils. Hypochlorous acid can react with a number of intermediate compounds forming a series of longer lived and more stable oxidant compounds. This array of oxidants is a formidable insult to both invading pathogens and host cells. The hydroxyl radical particularly causes peroxidation of lipid membranes and in the presence of transition metals such as copper and iron these membranes disintegrate, lose their integrity, resulting in increased permeability (15).

Another seemingly potent mediator in the lung injury seen with endotoxin induced ARDS is the inappropriately named platelet activating factor (PAF). This phospholipid, which is secreted by many mammalian cells and tissues, has been identified as 1-O-alkyl-2-acetyl-sn-glycero-3 phosphorylcholine. PAF aggregates and stimulates both leukocytes and platelets and contracts pulmonary artery and airway smooth muscle in many species (16,17). We have

recently successfully measured this compound in lung homogenates from both control and septic animals.

EXPERIMENTAL METHODS AND STUDIES OUTLINED.

The Model

The porcine model was used in all the ensuing experiments. Young swine weighing between 15-25 kgs were anesthetized with intramuscular ketamine hydrochloride 25 mg/kg and placed supine. Anesthesia was induced with sodium pentobarbital (10 mg/kg) and maintained with intermittent bolus pentobarbital as necessary. Following intubation with a cuffed endotracheal tube; they were paralyzed with continuous intravenous pancuronium bromide (0.2 mg/min) to permit mechanical ventilation with 0.5 FiO₂, 5 cm H₂O positive end expiratory pressure (PEEP) and 20cc/kg tidal volume at a rate which produced a PaCO₂ of approximately 40 torr at the beginning of the experiment.

Catheters were inserted into the left common carotid artery for monitoring systemic arterial blood pressure (SAP) and arterial blood gases, and into the right and left external jugular veins for infusion of Pseudomonas (Ps) and the therapeutic agents to be studied. A thermodilution Swan-Ganz catheter was passed through the right jugular vein into the pulmonary capillary and wedged in position with the balloon inflated. This was used to monitor pulmonary artery pressure (PAP), pulmonary wedge pressure (PWP), and thermodilution cardiac output. Cardiac output was converted to cardiac index (CI) by the formula:

$$CI = 0.112 \frac{CO}{BW^{2/3}}$$

where BW is the body weight in kg. Blood gases were measured with a blood gas analyzer (Instrumentation Laboratories, Model 113).

A 5 French femoral artery lung water catheter (American Edwards Laboratories, Model 96-020-5F) was passed into the lower abdominal aorta for measurement of extravascular lung water (EVLW). In this technique 10 ml of iced, green dye solution (2 mg indocyanine green dye in 10 ml 5% dextrose) were injected as a bolus through the proximal port of the Swan-Ganz catheter as blood was simultaneously withdrawn through the thermistor-tipped femoral artery catheter and a densitometer cuvette (Waters Instruments Inc., Model 402A) linked to a lung water computer (American Edwards Laboratories, Model 9310). The computer measured the mean transit times of the intravascular dye (MTD) and freely diffusible thermal component (MIT) as well as the cardiac output (CO). EVLW was calculated by the formula:

$$\text{EVLW} = \frac{\text{CO} (\text{MTD}-\text{MIT})}{\text{BW (kg)}}$$

Bronchoalveolar lavage was performed at 0 minutes 60 minutes and at 5 hours to harvest resident alveolar macrophages and PMNs and to provide alveolar lining fluid for biochemical studies including protein content.

Peripheral blood samples were be withdrawn at baseline (zero timepoint), 1 hour (i.e. directly after the Pseudomonas infusion) and at 5 hours (end-stage sepsis) and neutrophils were isolated using dextran sedimentation and Ficoll-sodium diatrizoate density gradient centrifugation. Assay of phorbol ester stimulated

production of oxygen dependent neutrophil products were performed as outlined below. The volume of blood withdrawn was determined by the peripheral white blood cell count measured 15 minutes prior to the designated time points. Similarly arterial blood samples were withdrawn at 15 minute intervals for estimation of tumor necrosis factor levels.

Pulmonary compliance measurements: Pleural pressure was measured by positioning an esophageal balloon (National Catheter) in the mid-esophagus where changes in esophageal pressure were most negative and interference in the pressure signal due to cardiac motion was minimized. Airway pressure was measured from a side port of the ventilator tubing just proximal to the endotracheal tube. Airway and esophageal pressure catheters were connected across a differential pressure transducer (Validyne MP45-4, range + 50 cm H₂O, Validyne Engineering Corp., Northridge, CA) for measurement of transpulmonary pressure. Exhaled volume was measured by connecting a pneumotachograph (Model 8805B; Hewlett-Packard, Waltham, MA) in line with the ventilator exhalation tubing which was then coupled to a flow transducer (Model 47304A; Hewlett-Packard, Waltham, MA). The airflow signal was sent to a respirator integrator (Model 8815A; Hewlett-Packard, Waltham, MA) for volume determination. Volume was electronically plotted against both airway and transpulmonary pressures (X-Y Recorder Module; Warren E. Collins Inc., Braintree, MA). Pressure transducers were calibrated daily using a water manometer; the pneumotachograph and volume integrator were calibrated using a precision syringe.

For measurements of dynamic compliance (C_{dyn}), the pneumotachograph was placed at the exhalation port of the Harvard Ventilator for volume determination. A minimum of eight tidal volumes occurred before each dynamic compliance measurement to assure stabilization at functional residual capacity (FRC). Dynamic compliance was determined by the formula:

$$C_{dyn} = \frac{\text{Tidal Volume}}{\text{Peak Inspiratory Pressure} - \text{End Expiratory Pressure}} \times \text{Weight in kg.}$$

Static lung compliance (C_L) was determined by briefly connecting the animal to a pressure ventilator (Bird, Puritan-Bennet Corp.). The pneumotachograph was placed at the distal end of the exhalation tubing in this system. A hand operated, spring-loaded, on-off valve (Model BE137; Instrument Industries Inc., Bethel Park, PA) was introduced mid-way between the endotracheal tube and the pneumotachograph to halt exhaled airflow at 0.5 sec intervals. Since the exact total lung capacity in these animals was unknown, the lungs were inflated with pressurized O_2 to 25 cms H_2O pressure and then permitted to deflate passively. Static pressure/volume measurements were obtained by allowing exhalation to occur stepwise until functional residual capacity (FRC) was reached. Intervals of at least 0.5 seconds were inserted between each exhalation burst to ensure that pressures had stabilized. Each compliance measurement included at least eight data points throughout exhalation. Static lung compliance was computed from

the line of best fit applied to the pressure-volume curve by the following formula:

$$C_l = \frac{\text{Change in Lung Volume}}{\text{Transpulmonary Pressure/Weight in kg.}}$$

Static compliance of the respiratory system, (lung and chest wall combined) commonly measured in intensive care unit patients was not measured in this study. Measurement of C_l is independent of airway and chest wall contributions, and is specific for pulmonary parenchymal elasticity. C_{dyn} was measured to assess whether it would accurately reflect changes in C_l .

A less lethal model than that used previously is at present being employed. Live *Pseudomonas aeruginosa* (PAO strain, 5×10^8 CFU/ml at 0.3 ml/20kg/min) was administered for 1 hour rather than by continuous infusion. In Ps control animals this has been shown to produce a marked physiological deterioration, representative of acute ARDS, resulting in an immediate significant increase in PAP which persists throughout the entire duration of the experiment. SAP shows a progressive decline as does CI and PaO_2 . EVLW and SI become significantly elevated when compared to saline controls. We have already established in previous studies that treatment with ibuprofen alone attenuates the early rise in pulmonary artery pressure and the fall in arterial oxygen tension while having no effect on lung water. It is well established that these effects are due to blockade of cyclooxygenase metabolites. Combination treatment of cimetidine 150 mg, diphenhydramine 10 mg/kg and ibuprofen 12.5 mg/kg (CID) given i.v. at 20 and 120 minutes after

continuous pseudomonas infusion significantly improves all the physiological and hemodynamic parameters in the early stages of lung injury in the model with significant improvement in the hypoxemia, early pulmonary hypertension and pulmonary microvascular injury seen in Ps controls. With this information in mind these studies were repeated in the less severe model now in use in our laboratory. In these experiments the emphasis was on the production of both long and short lived neutrophil oxidants and on compliance changes and their attenuation following treatment.

Neutrophil oxidant generation studies.

Many investigators have established that human and various animal species produce toxic oxygen metabolites from neutrophils in vitro. Neutrophils become activated by contact with a soluble or particulate stimulus such as live bacteria, endotoxin (lipopolysaccharide) and undergo a respiratory burst with subsequent production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). We performed studies to document and properly characterize the kinetics of O_2^- release in addition to the total amount of these metabolites released from pseudomonas-primed porcine neutrophils. Neutrophils are separated from the pre- and post-injury blood samples and adjusted to a known concentration of cells per milliliter. For kinetic studies pre-injury and post-injury neutrophils are stimulated with phorbol myristate acetate (PMA) (a known white cell activator) in a dual beam spectrophotometer in the presence of cytochrome C at 550 nm. The

rate of superoxide dismutase inhibitable O_2^- production per minute is directly proportional to the rate of cytochrome C reduction.

In order to determine the direct stimulatory effect of live organisms upon neutrophil free radical generation without opsonization by serum factors and to eliminate the effect of endogenously produced inflammatory mediators upon reactivated oxygen intermediate production a series of in-vitro studies were performed. In these studies, arterial blood (25ml) was obtained from healthy young swine (n=5) into plastic syringes containing EDTA (0.1 ml, 15% EDTA/10 mls whole blood) at 0 min and 60 min following the 60 min infusion of 0.9% NaCl and neutrophil isolation performed as above. Superoxide anion production following PMA stimulation was assessed immediately following isolation of neutrophils from both samples. Neutrophils were then admixed in a ratio of 1:100 with live bacteria and incubated for 60 minutes at 37°. Controls for these studies contained no bacteria but the identical number of neutrophils incubated under the same conditions. At the end of the 60 minute incubation, cytochrome C and PMA were added to reaction mixtures and the change in optical density was followed continuously over a ten minute period as for the ex-vivo PMNs studies described above. The nanomolar extinction coefficient of 0.0211 was used to quantify cytochrome C reduction. The production of superoxide anion was calculated for each minute and plotted against time. Rates are expressed as nmols of O_2^- /min/ 10^6 PMNs.

Compliance Studies.

Static and dynamic pulmonary compliance are important parameters in human ARDS as well as animal ARDS models. A method for estimating these parameters in the porcine model has recently become available to this laboratory as outlined above. We are at present examining static and dynamic compliance in the injured animals compared to controls, and will then compare the effects of various treatment modalities on these parameters. Initially, ibuprofen therapy was studied alone, and this was followed by a study utilizing CID therapy (see results section).

Ibuprofen studies.

To date this non steroidal anti-inflammatory drug has been proven to be the most effective drug in ameliorating the derangement in hemodynamic parameters in ARDS. Its effects have been shown to be due to the blockade of cyclooxygenase metabolites of arachidonic acid, in particular thromboxane. There are data from in-vitro studies to suggest that ibuprofen may have an effect on neutrophil oxidant generation by altering the intracellular NADPH-oxidase system. We proposed to examine the production of neutrophil oxidants in both treated and untreated animals and to demonstrate any effect of this drug.

Studies with combination therapy cimetidine, diphenhydramine and ibuprofen (CID).

This drug combination has been shown to be effective in preventing circulatory collapse in sepsis-induced ARDS in animal models. In-vitro work has shown that histamine is an inhibitor of neutrophil oxidant release. Therefore treatment of circulating neutrophils with anti-histamines should release this inhibition. We wished to examine the effects of antihistamines on neutrophil oxidant generation in this in-vivo situation and relate these data to hemodynamic changes.

Long-lived oxidant estimation.

Current evidence suggests that a system composed of H_2O_2 , the azurophilic granular enzyme myeloperoxidase (MPO) and a halide (H_2O_2 -MPO-Halide) constitute a source of another group of potent toxic oxidants produced from neutrophils. The hypohalous acids (HOCL, HOBR, HOI) and monochloramines are compounds active in the intracellular killing of micro-organisms within phagocytic vacuoles. These compounds are also secreted by stimulated PMN and may attack local targets or modify neutrophil proteolytic enzyme behavior. In fact, it is widely believed that this system is the most potent oxidant system associated with neutrophil cytotoxic function. The role of the long lived oxidants is not well defined. However, the biological reactivity and cytotoxic potential of the powerful oxidant hypochlorous acid and its chloramine derivatives

(e.g. taurine chloramine) suggest that these active oxidants play an important role in the inflammatory response and in host defense. Moreover, mounting evidence implicates products of this system not only as direct cytotoxic agents but as potent modulators of the neutrophil inflammatory response. The known actions of the long lived oxidants not only include antibacterial activity but also, inactivation of alpha-1-proteinase inhibitor allowing unrestricted elastase mediated tissue damage (particularly in the lung), as well as activation of the latent proteinase, collagenase, generating a full proteolytic enzyme which specifically destroys collagen. Finally they permit oxidation of chemical compounds such as certain drugs, industrial pollutants and environmental chemicals to damaging electrophilic free radical forms. The importance of this system is that it allows neutrophils to convert the short lived non-specific effects of primary oxygen radicals into highly specific long-acting effects by producing compounds which modify cellular or plasma constituents critical to the inflammatory response. This laboratory has recently used a continuous spectrophotometric assay which can determine the amount and kinetics of hypohalous acid production by phagocytic cells.

The assay employs the ability of the amino acid, taurine, to act as a scavenger of the hypohalous acids, the resulting taurine halamides can oxidize 5-thio-2-nitrobenzoic acid (TNB) to the disulfide, 5-5'-dithiobis (2-nitrobenzoic acid DTNB) and the change in optical density measured at 412nm allows estimation of the production of hypohalous acids.

Endothelial cell line and flow cytometry studies.

It is unclear whether activation of neutrophils by prior exposure to phagocytic stimuli also alters the adherence and cytotoxic potential of these cells. By utilizing a cultured endothelial cell line it is possible, to determine the ability of artificially stimulated neutrophils to adhere to, or to cause cell lysis of, cultured endothelial cells (pulmonary artery endothelium or, preferably, pulmonary microvascular cells). In conjunction with this, we are studying the regulation of adherence glycoproteins on the neutrophil surface by flow cytometry. It is generally agreed that a receptor interaction is the means by which neutrophils adhere to the vascular endothelium, and most investigators believe that the most important receptor is the CD-18 glycoprotein. We are interested in studying the physiology of this receptor during sepsis in the porcine model. We hope to be able to block the receptor using a monoclonal antibody, anti CD-18, 60.3. To date preliminary flow cytometric data suggest an upregulation in the receptor status. We believe this correlates with the profound peripheral neutropenia which develops in this ARDS model following the infusion of live organisms (Fig 10). It is eventually hoped that substantial quantities of this monoclonal antibody will become available to us thus enabling infusion of therapeutic doses into study animals.

Tumor necrosis factor (TNF) study.

It is now widely believed that TNF is the major inflammatory mediator involved in the final common pathway for all types of ARDS (i.e. traumatic, septic etc). TNF is thought to be produced by the mononuclear cells, in particular monocytes and tissue macrophages. In the past year we have measured levels of TNF in the porcine model using a bioassay in which the cytotoxic potential of serum factors to murine L-929 cells is quantified.

RESULTS

Oxidant generation.

Fifteen pigs were studied, 5 controls and 10 septic animals. Figure 1a and 1b show the results of the kinetic assays for superoxide production from neutrophils following stimulation with PMA. The results are depicted graphically as nmol of superoxide anion/ 10^6 PMN/min. This kinetic display of neutrophil function allows examination of the potential priming of the cells to produce oxidants. Figure 1a shows the results obtained from cells harvested from control (C) animals at 0 and 60 minutes. This figure clearly shows no difference between cells harvested at these time points during the course of the experiment. This means that experimental manipulation, surgery and prolonged ventilation using high inspired O_2 concentrations did not effect the PMNs ability to produce oxidants. In contrast however, PMNs from animals which received an infusion of live *Pseudomonas* organisms demonstrated marked enhancement of their abilities to produce oxidants, particularly in the first 2-3 minutes following stimulation, as indicated by a marked leftward shift in the curve. This essentially suggests that cells which remain in the peripheral blood after the onset of a profound bacteremia, as is the case in this model, can mount an effective antibacterial challenge (Fig 1b) and are "primed" to produce oxidants in greater quantities and at faster rates.

In-vitro simulation (n=5): The kinetic production of superoxide anion from neutrophils in this in-vitro study are

depicted in figure 2a and 2b. Cells harvested from the animals at 0 and 60 minutes (i.e. before and after saline infusion) did not demonstrate any difference in production of O_2^- over the ten minute assay period. In contrast the same cells incubated for 60 minutes with live *Pseudomonas* showed a marked increase in O_2^- production compared to cells from the same sample which were incubated in buffer alone. The maximum rate of production from the Ps incubated cells was 2.17 nmol/min/million cells at 2 minutes which is a 2.34 fold increase ($p < 0.01$) over buffer incubated cells. Addition of ibuprofen to septic animals resulted in a dramatic reversal of superoxide anion generation upregulation. As can be seen from figure 3a,b and c, the generation of superoxide anion in the ibuprofen treated animals parallels that of the control group. Maximum rate of production at all time points has the same prolonged lag time as for control animals. However treatment with CID therapy causes a release of this attenuation (preliminary data, not shown), treated cells are now able to produce levels equivalent to those of cells from septic unprotected animals.

Compliance studies.

Dynamic compliance (C_{dyn}) remained at or near baseline levels throughout the study in control animals (Fig 4). In group Ps, however C_{dyn} decreased to 73% and 67% of baseline values at 30 and 60 minutes following the onset of *Pseudomonas* infusion. Values in *Pseudomonas* infused animals decreased to 36% - 45% of baseline from 150 mins to the end of the study. C_{dyn} in septic animals became

significantly decreased compared to controls ($p < 0.01$) at 30 mins and remained significantly lower throughout the study.

Static lung compliance (C_L) remained at or near baseline levels throughout the study in controls (Fig 5). In septic animals, C_L decreased to 61% and 54% of baseline values at 30 and 60 minutes respectively. Values thereafter remained between 40% - 57% of baseline. Measurements in septic animals were significantly lower than controls from 30 mins until the end of the study ($p < 0.01$). The effect of ibuprofen on the compliance changes in septic animals can be seen in figures 6 and 7, where attenuation of both static and dynamic compliance are demonstrated. Preliminary data would suggest that CID treatment also causes significant attenuation of the fall of pulmonary compliance.

Long lived oxidants.

The production of hypochlorous acid from septic neutrophils was increased over that of control animals (Fig 8); however, the addition of ibuprofen attenuated this increase (Fig 8) and preliminary data suggest that pigs treated with triple therapy sustain this attenuation (data not shown).

Endothelial cell line and flow cytometry studies.

Data from these studies to date are only preliminary but do support the in-vivo data described above. There is upregulation of neutrophil surface receptors as measured by flow cytometry and endothelial cell line studies in-vitro demonstrate increased adherence capacity.

Tumor necrosis factor (TNF) study.

Figure 9 clearly demonstrates the high levels of TNF which are released following the infusion of live organisms into these animals. This figure depicts % TNF-induced cytotoxicity of the murine fibroblast cell line in plasma harvested from the two groups.

CONCLUSIONS.

The lung injury in ARDS is mediated by many different substances, some of which may yet be unknown. Histamine, prostaglandins, oxygen free radicals, small molecular weight biologically active lipids, interleukins, tumor necrosis factor and proteolytic enzymes have been implicated in the injury in in vitro and in vivo experiments. We have already established in the porcine pseudomonas model that treatment with a combination of H₁ and H₂ blockers and a cyclooxygenase inhibitor can attenuate all phases of the injury, particularly the early phase of pulmonary hypertension, as well as the late permeability phase.

We have established that cyclooxygenase inhibitors prevented the rise in TxB₂ and in PAP. It appears that thromboxane plays an important role in PAP mediated, endotoxin and pseudomonas lung injury.

In vivo studies with oxygen radical scavengers in this laboratory have been inconclusive and generally disappointing. It is becoming clear from in vitro work that oxygen radicals do have a role in the injury, but how this can be prevented is not apparent. The administration of exogenous radical scavengers in this model system have not altered the injury in any significant way, although some success has been reported in rats, guinea pigs and sheep. Part of the difficulty in assessing these compounds is that there is no good marker of oxidant injury in serum or in

tissue. However, the indirect methods used in this laboratory and outlined in this report show that the potential of circulating phagocytes to cause cellular damage can be adequately assessed.

We have established in this model that pseudomonas "primed" neutrophils produce superoxide radicals at a much greater rate than in control animals, thus implicating neutrophils as a major injury cell in the early phase of the process. However, it appears from some preliminary results that alveolar macrophages may also be implicated in the injury but that priming of these cells because of their position takes longer than neutrophils. It may be that macrophages, in addition to producing interleukins and tumor necrosis factor, also produce superoxide and its metabolites and may be involved in the later stages of alveolar-capillary injury.

Studies are ongoing with compounds which prevent neutrophil margination and adherence (CD-18, monoclonal antibodies) to the endothelium and thus may prevent release of inflammatory mediators. It is hoped that compounds such as this will limit the extent and the severity of injury. Assays of tumor necrosis factor and the interleukins are also ongoing. Tumor necrosis factor increases significantly shortly after the onset of pseudomonas infusion in the model and probably plays an important role in the early phase of the injury.

Production of both short and long lived oxidants are elevated in septic animals and this elevation is attenuated to a large extent by ibuprofen. However CID therapy appears to counteract this important therapeutic effect despite a further improvement in

hemodynamic parameters. Explanation of this apparent paradox will require further work.

Both static and dynamic compliance decreased dramatically after onset of bacterial infusion, but this was attenuated by administration of ibuprofen and initial data demonstrates an increased benefit from using CID. Compliance measurements will remain an important physiological parameter in the future evaluation of therapeutic intervention in porcine pseudomonas ARDS.

RECOMMENDATIONS

ARDS in both civilian and military patients carries a considerable mortality. The syndrome often occurs in previously fit individuals who undergo direct pulmonary as well as non-pulmonary injuries. It has been established that the lung injury seen in ARDS is due to many inflammatory mediators. Elucidation of these mediators and pathways are therefore likely to be necessary for adequate pharmacological intervention and treatment of the syndrome. We have established in animal models that a combination of H₁ and H₂ blockers and a cyclooxygenase inhibitor significantly improve all parameters but do not completely abolish the syndrome. The data referred to in this report provides more evidence of the beneficial effects of these therapies. The presence of significantly elevated TNF levels in this model suggest that the use of immunomodulation (monoclonal antibodies to TNF) in this condition may provide important further advances. Continued efforts at elucidation and identification of the various inflammatory mediators, particularly substances secreted by neutrophils and macrophages and pharmacological blockade of their actions will be the purpose of further research in this laboratory.

Figure 1a

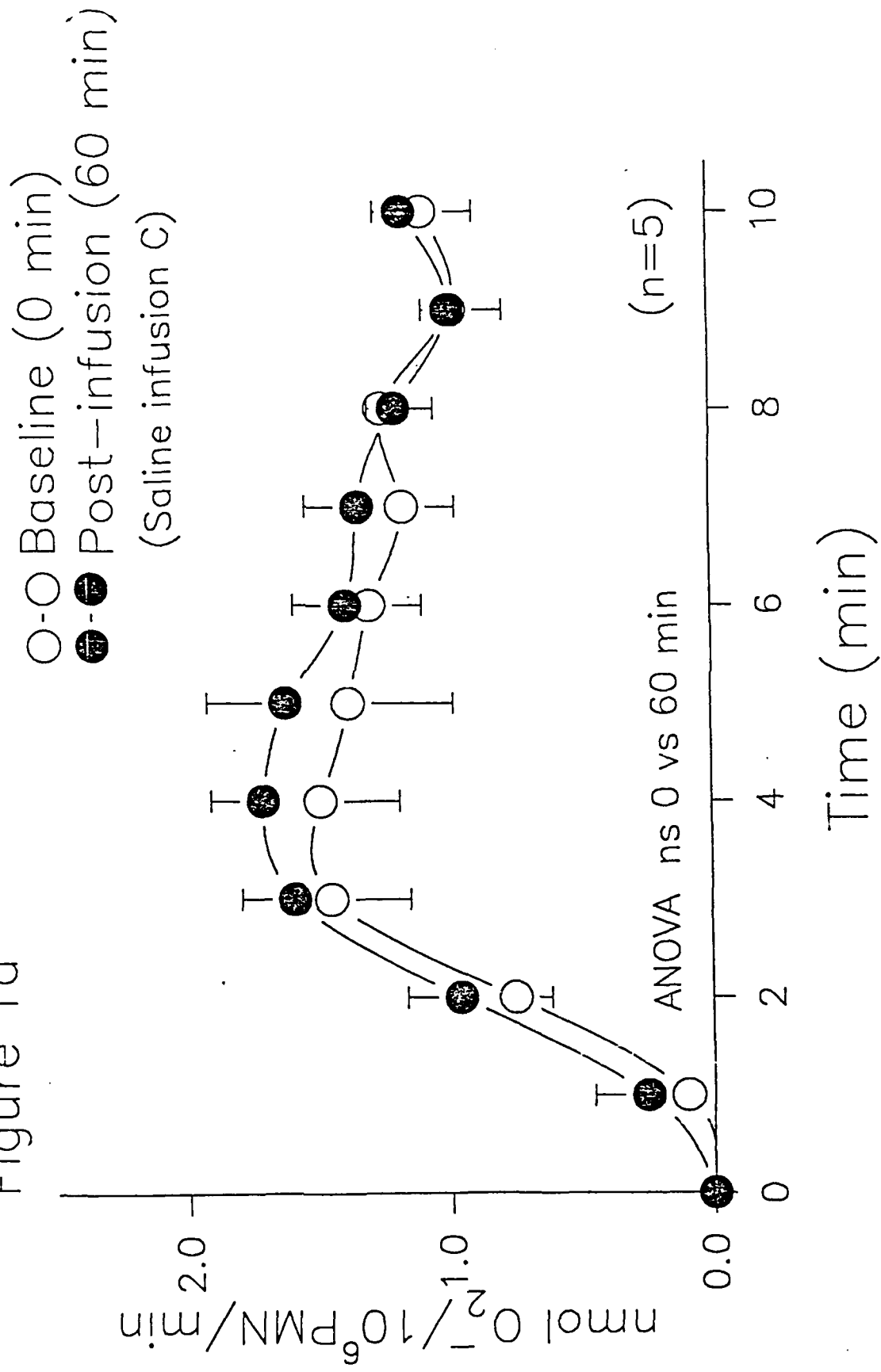


Figure 1b

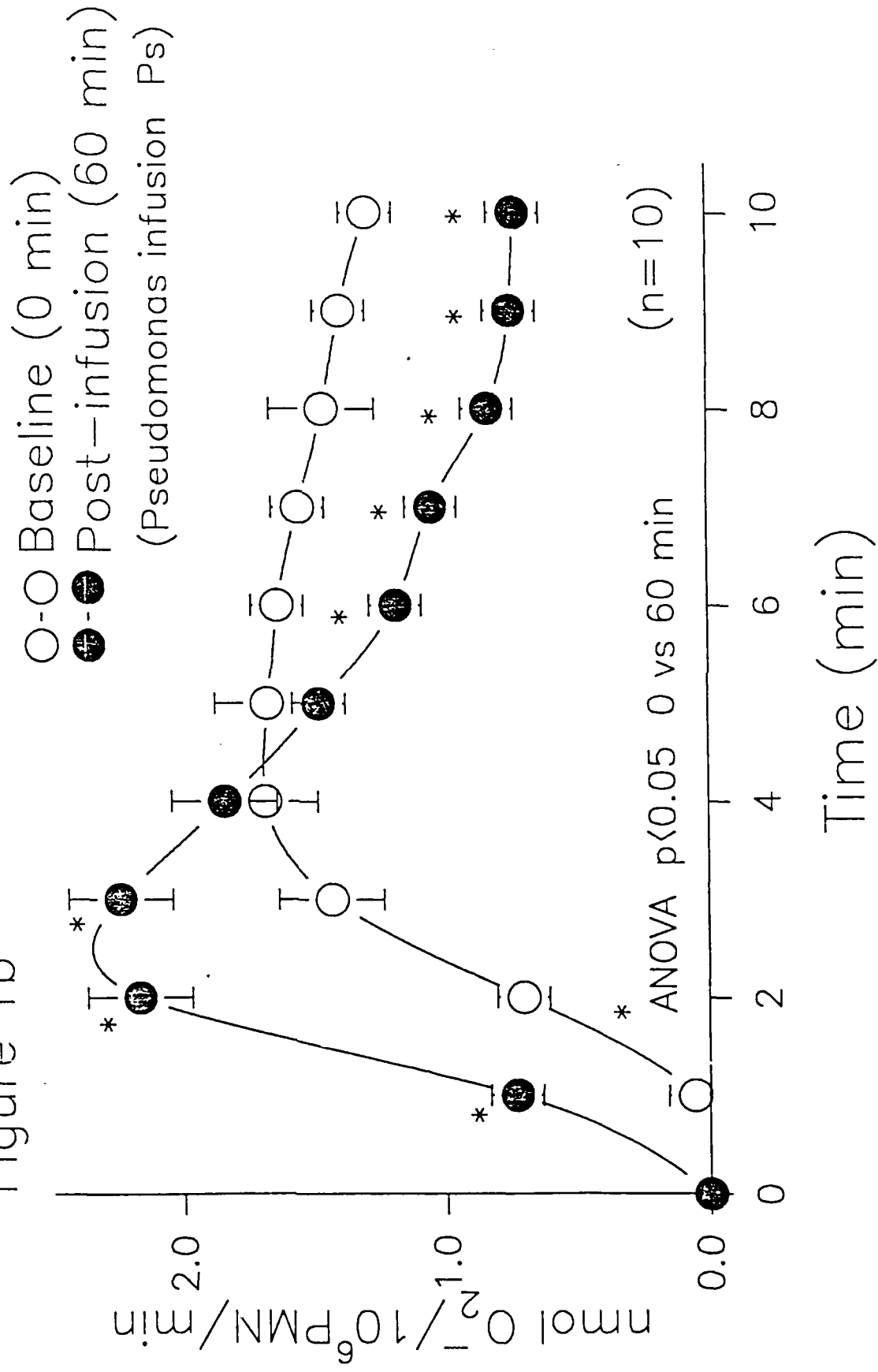


Figure 2a
(Saline infusion)

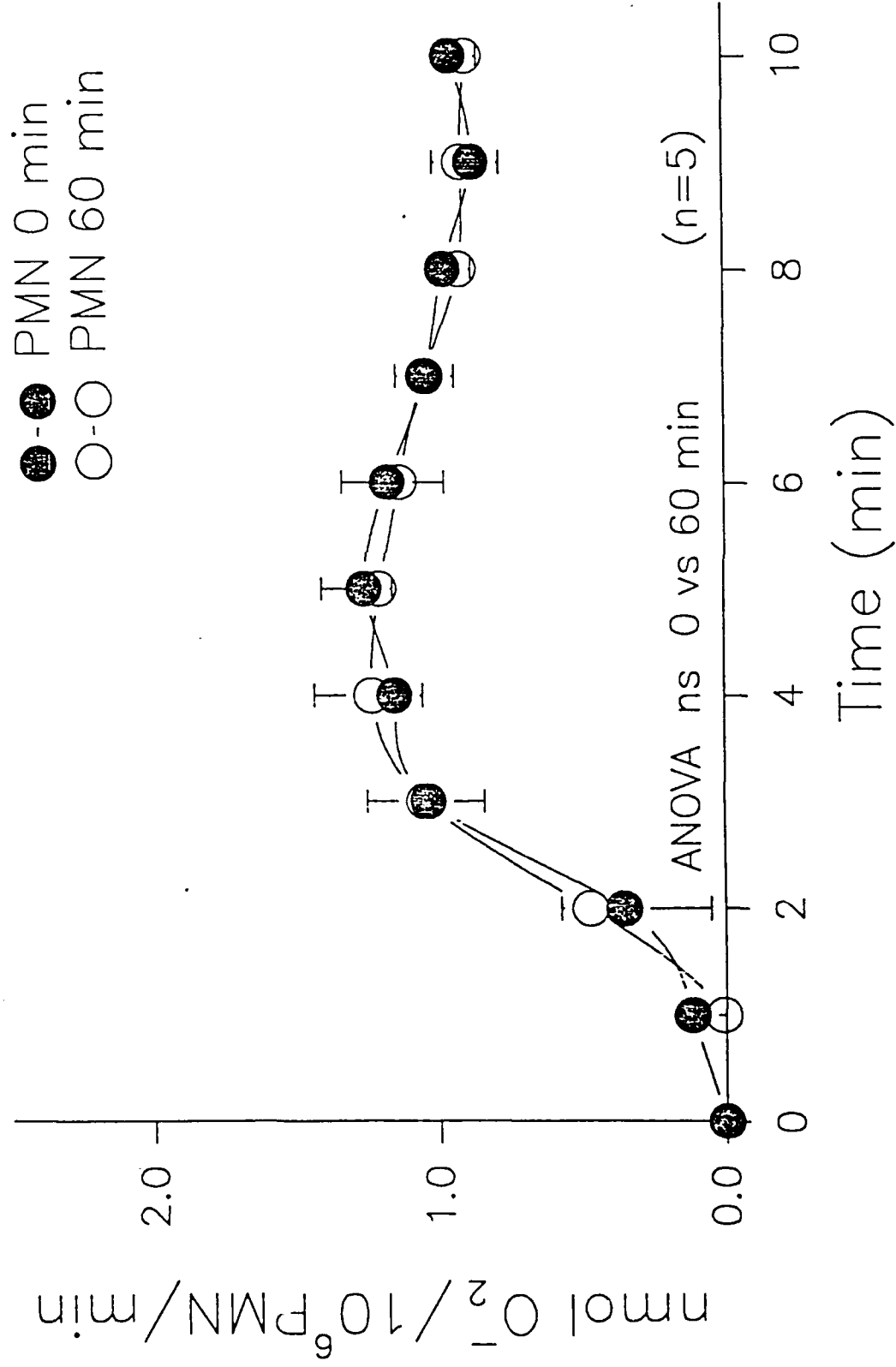


Figure 2b

(In-vitro Incubation)

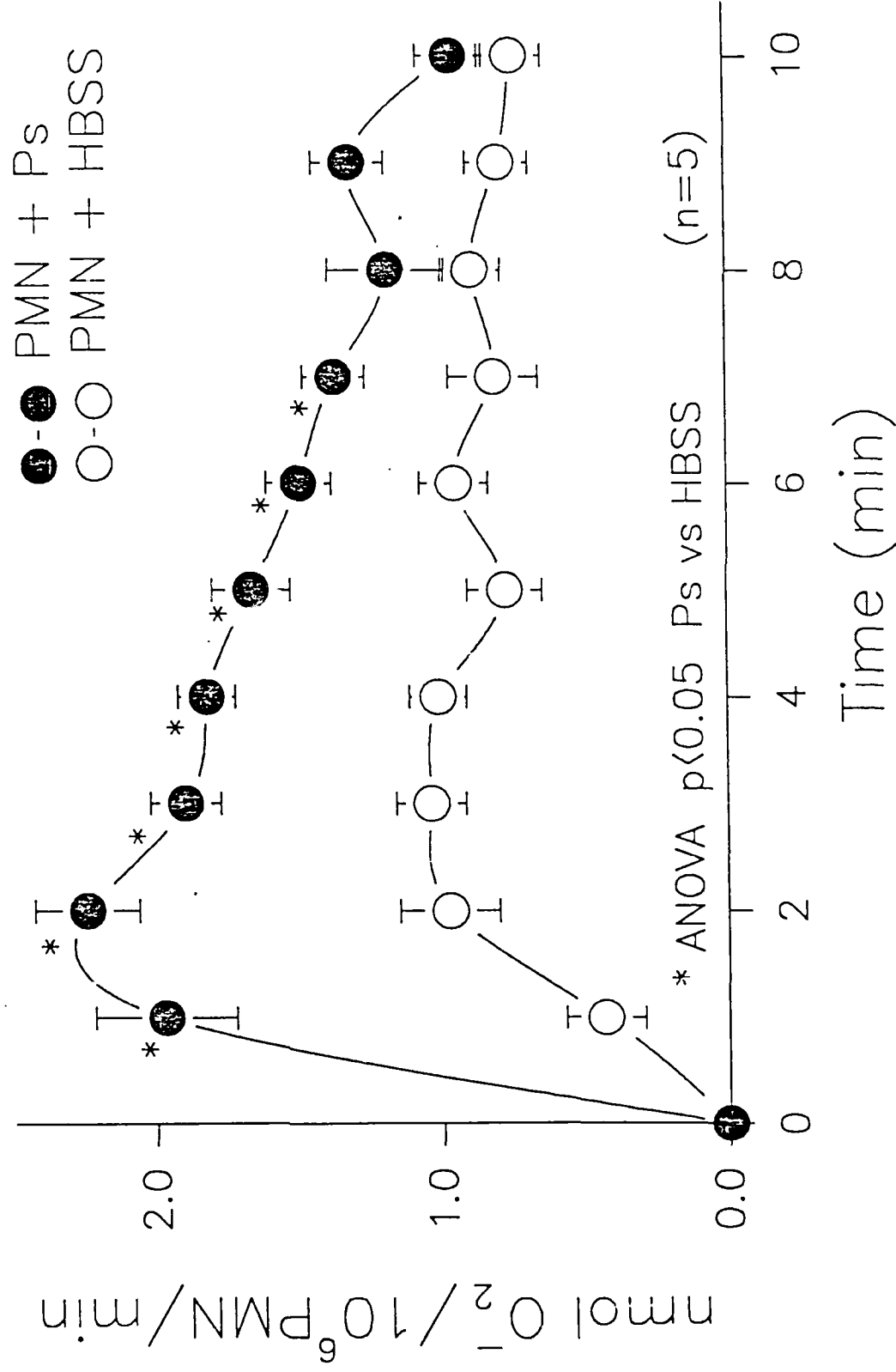
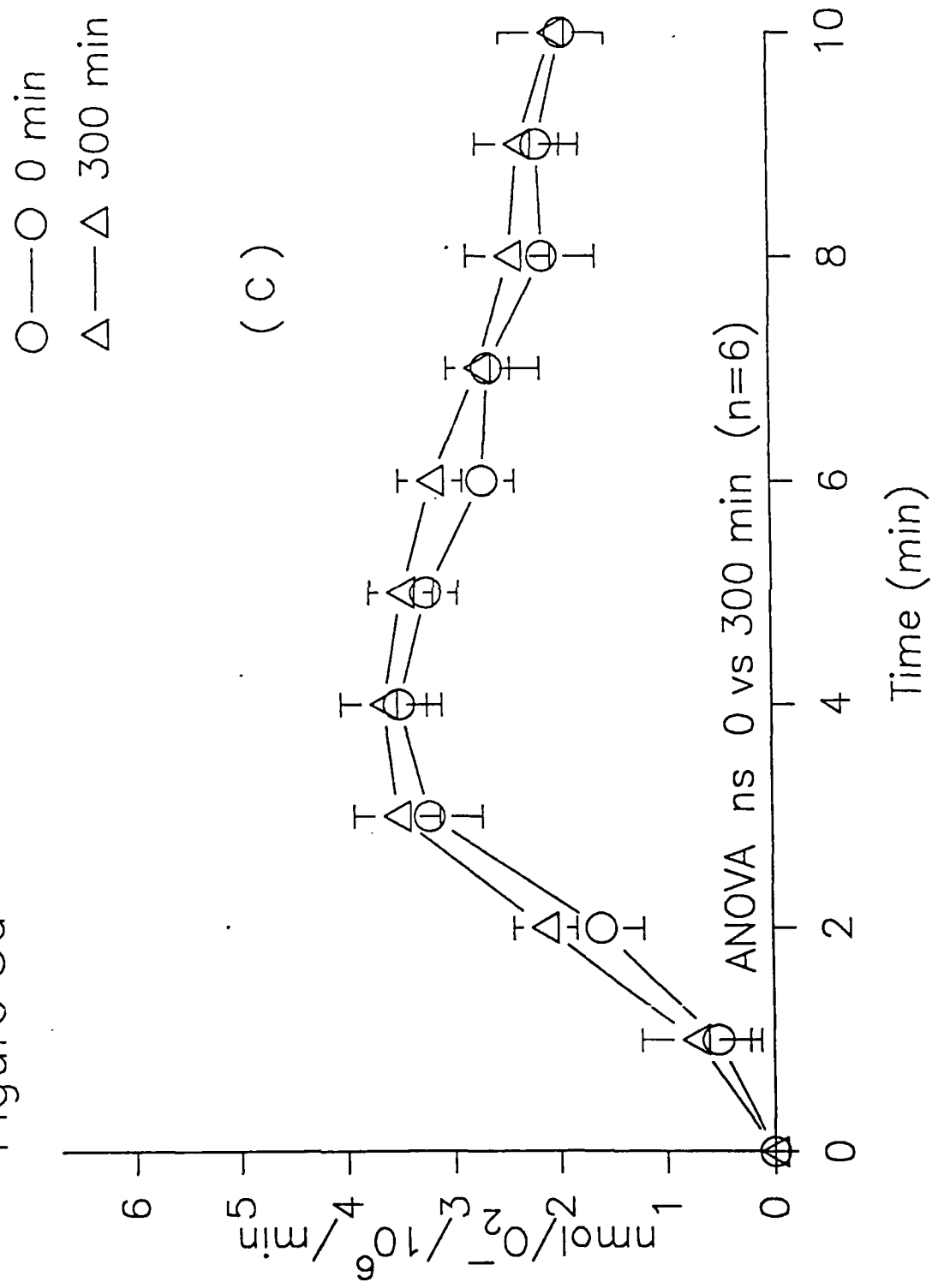


Figure 3a



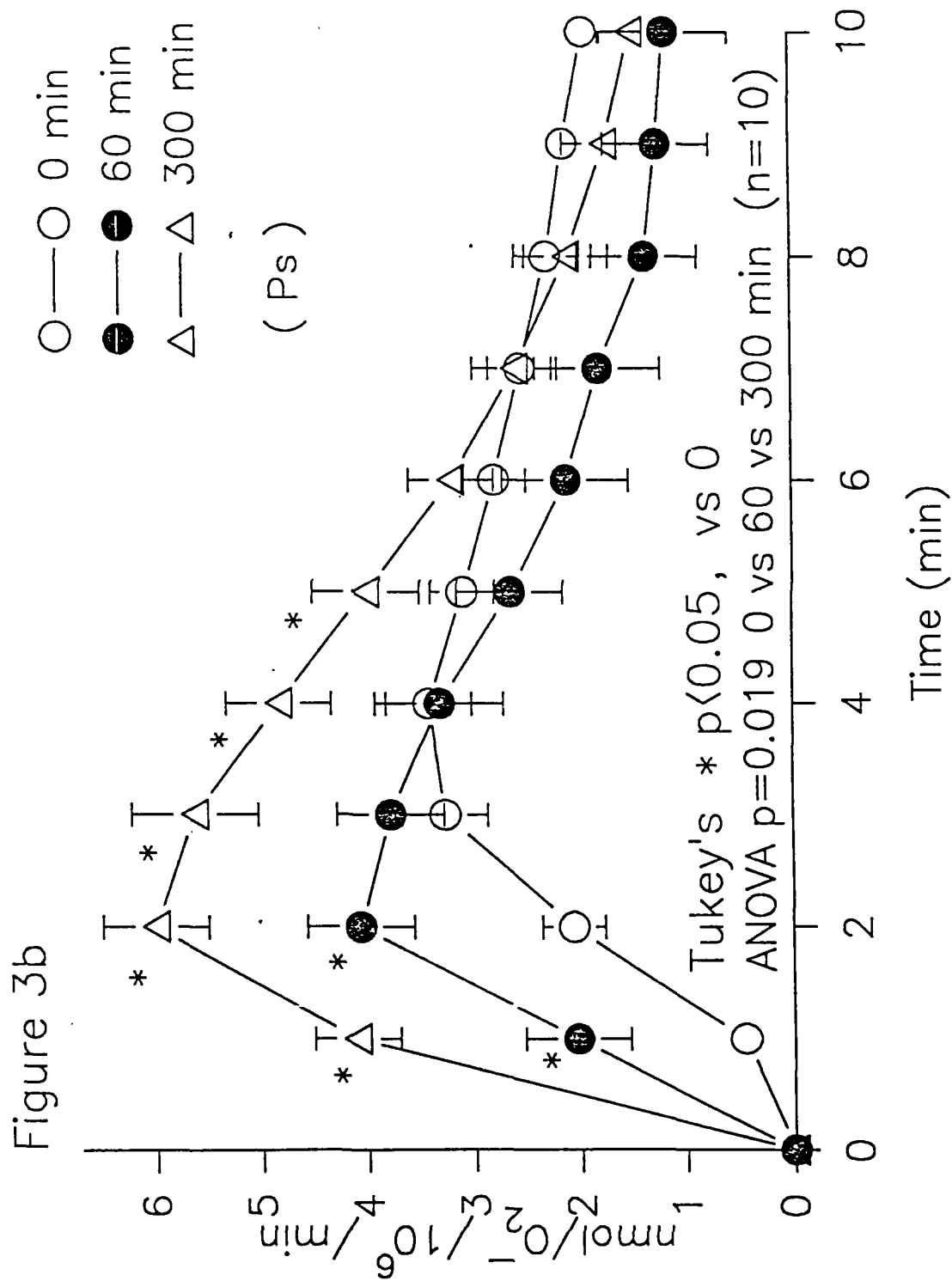
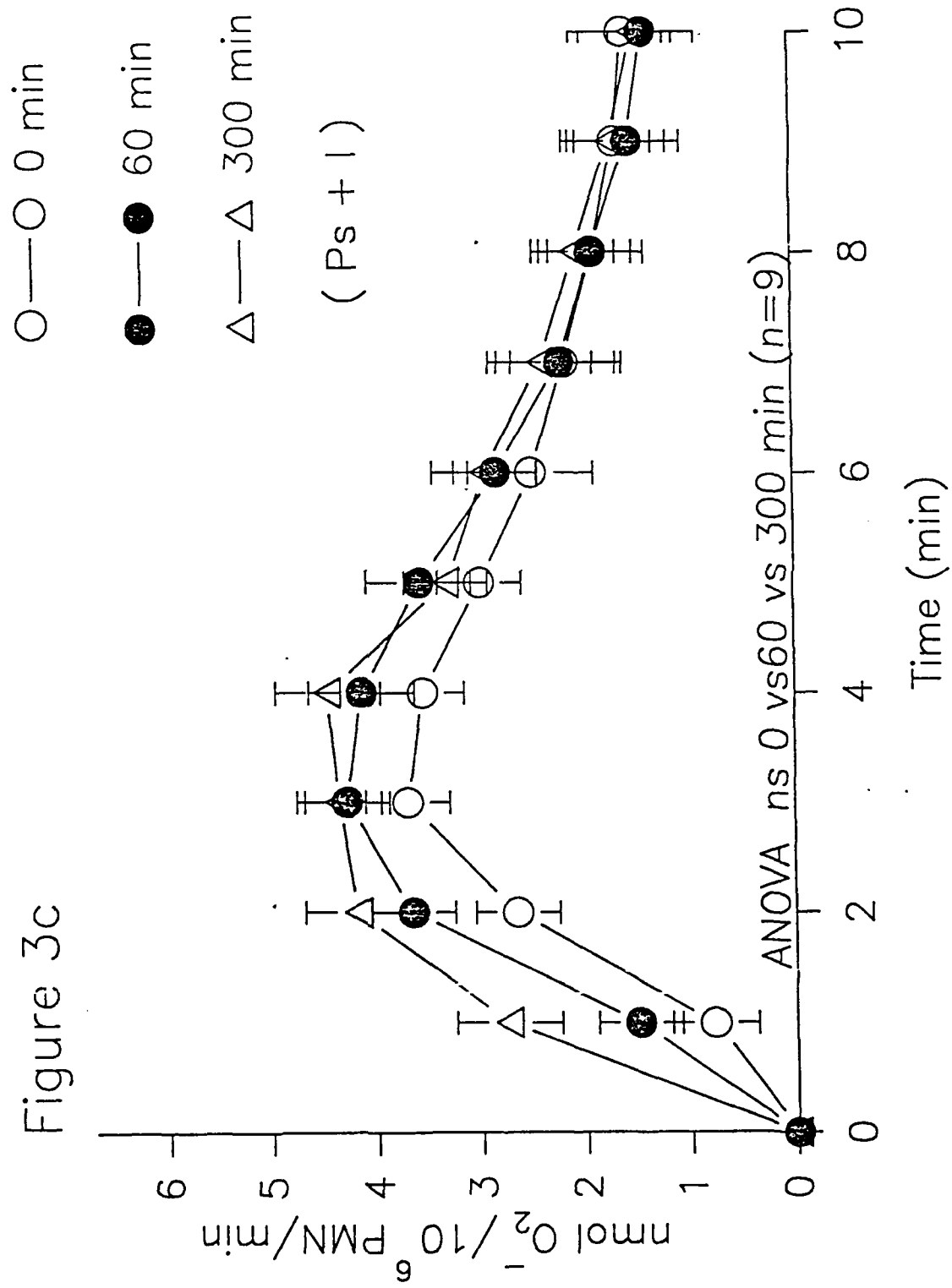


Figure 3b

Figure 3c



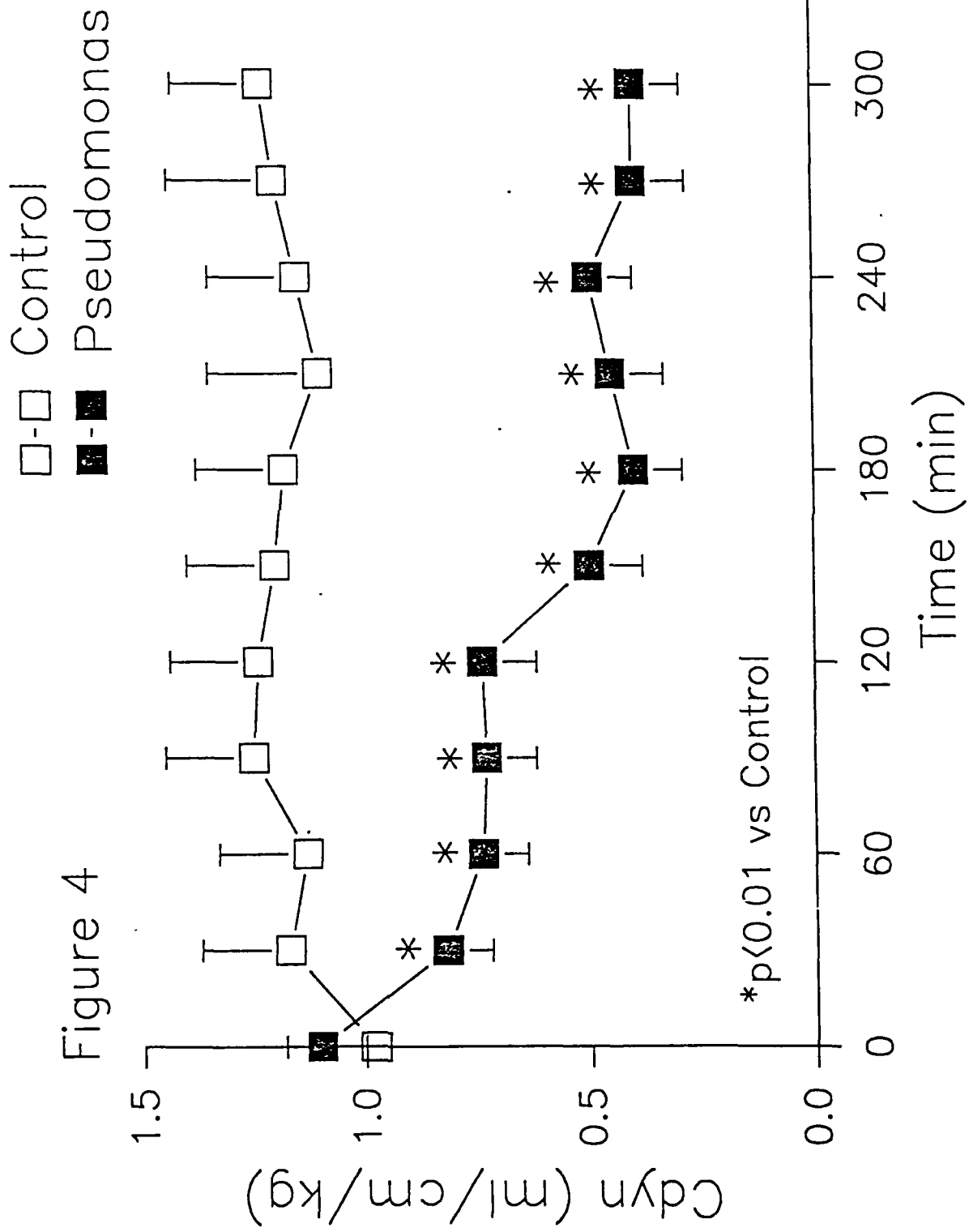


Figure 5

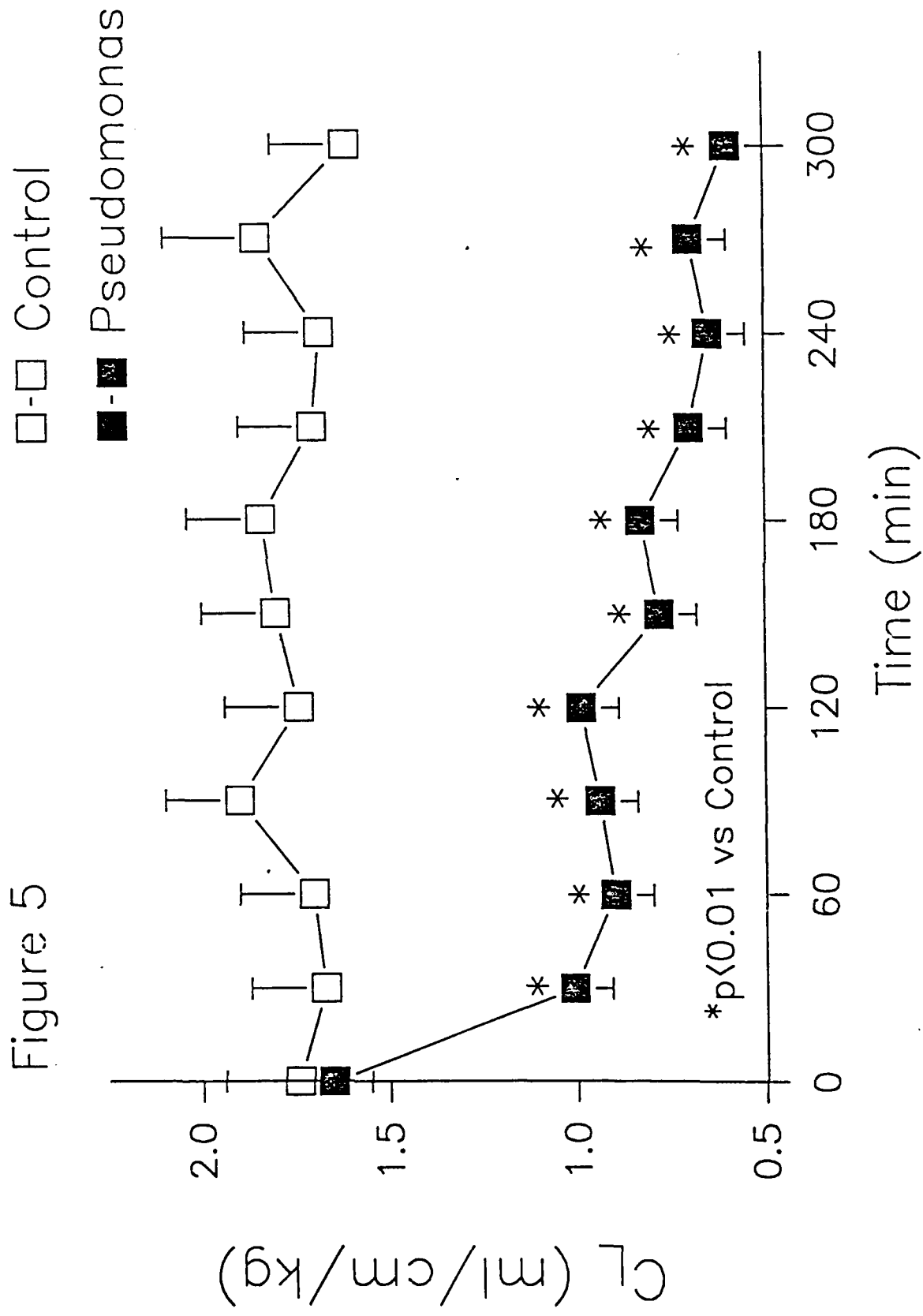
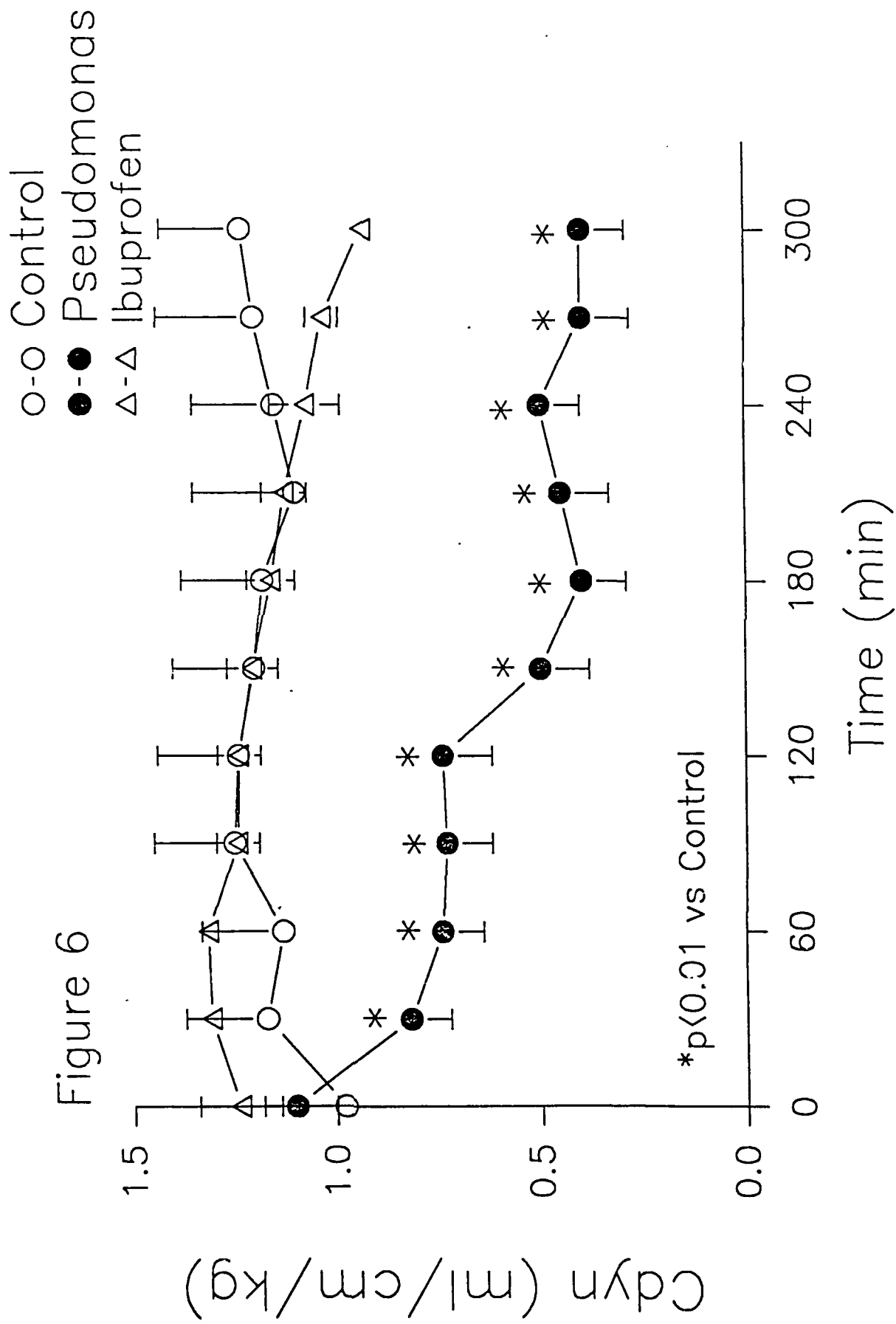


Figure 6



o-o Control
 ●-● Pseudomonas
 Δ-Δ Ibuprofen

Figure 7

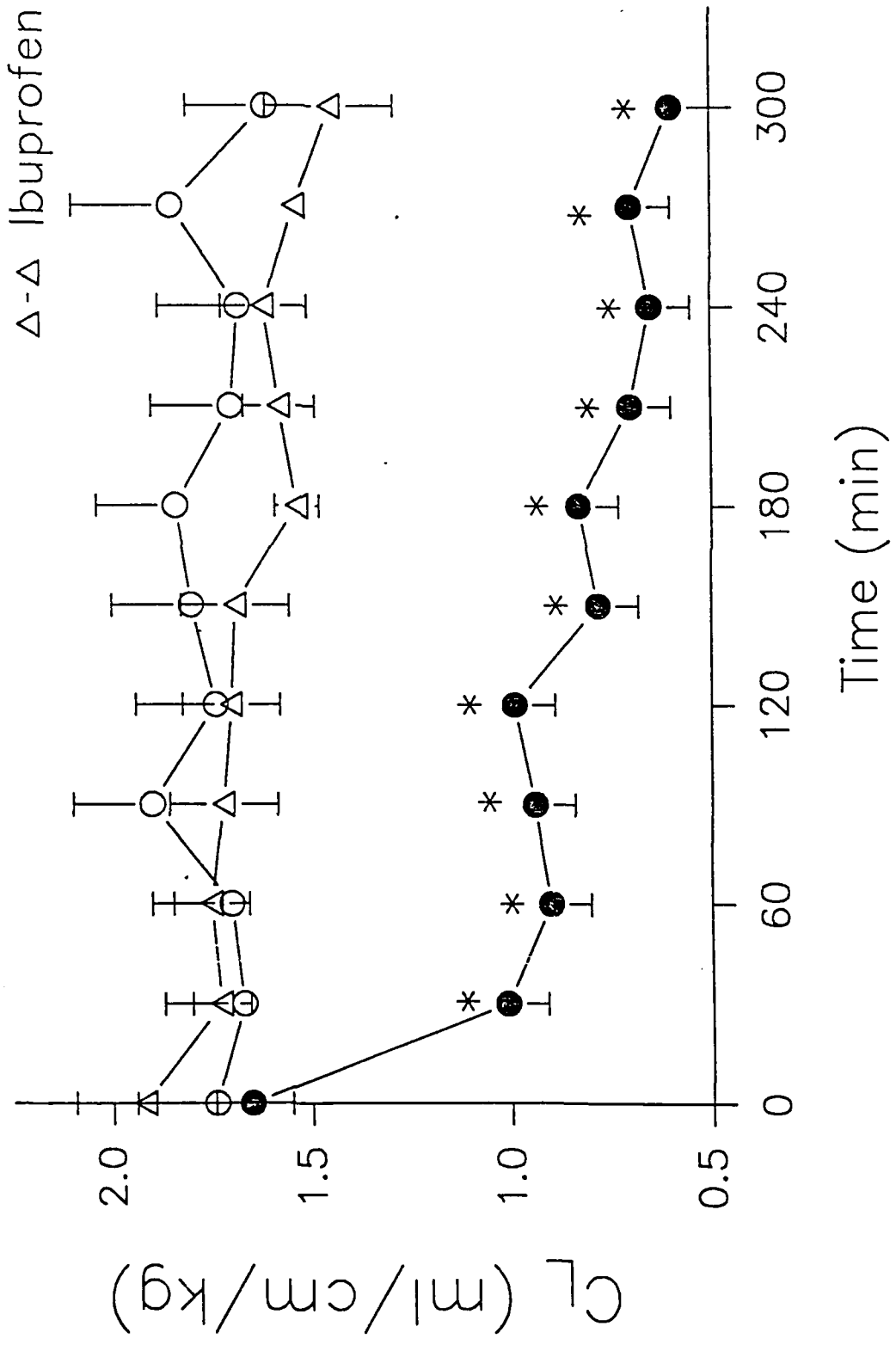


Figure 8

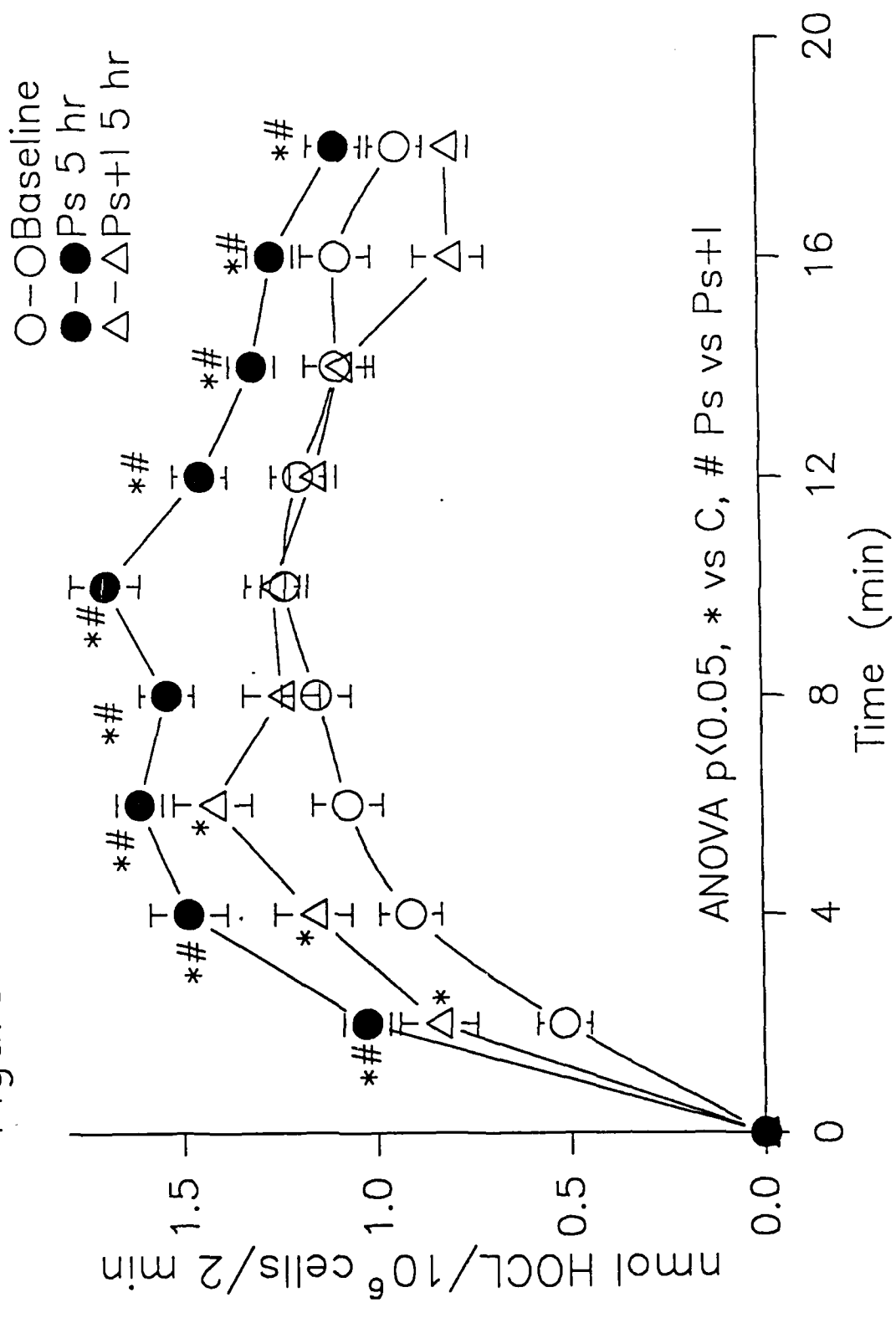
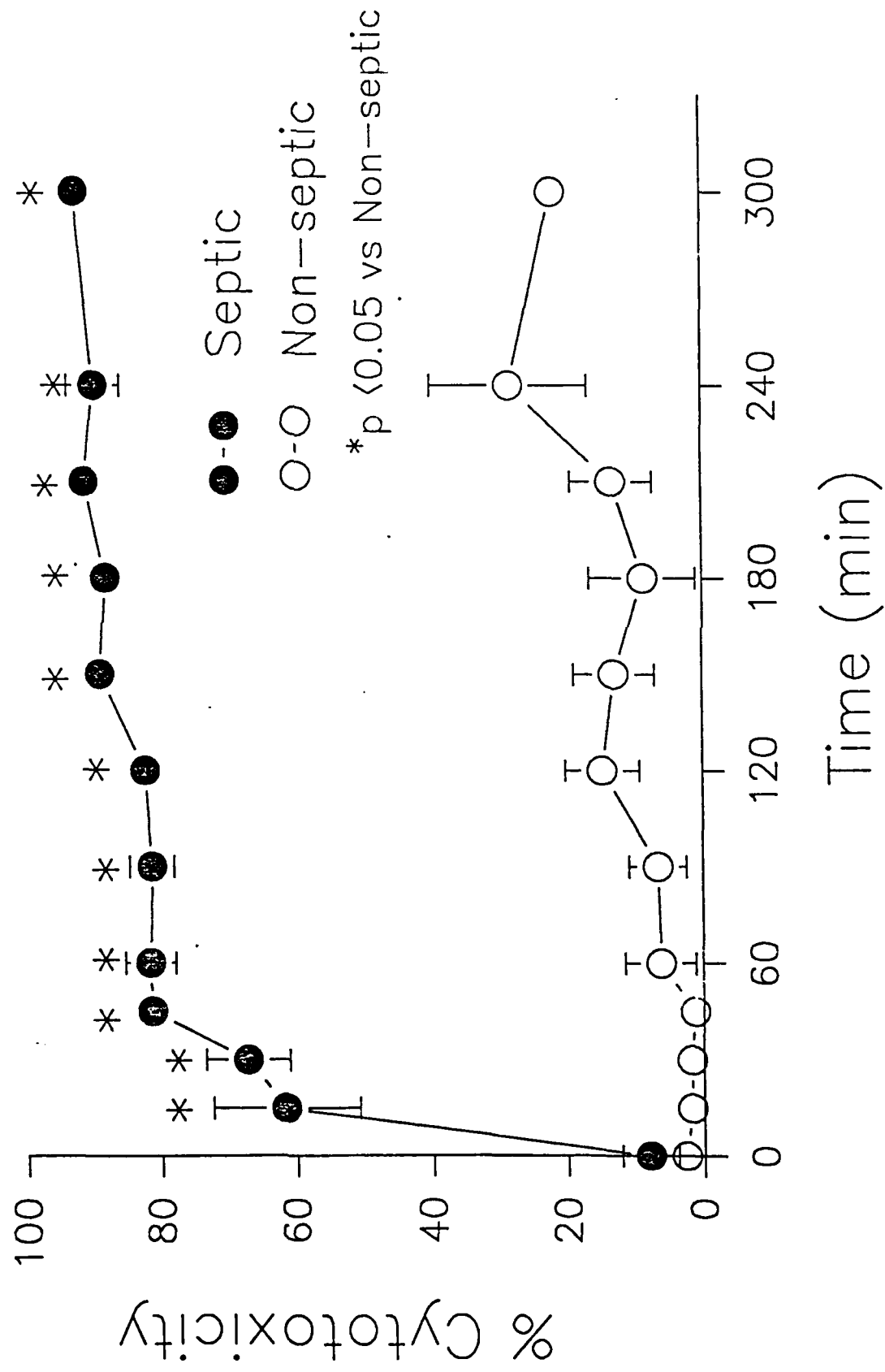


Figure 9



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