# EFFECT OF INHALED CATIONIC POLY-PEPTIDES ON RESPIRATORY MECHANICS IN THE ISOLATED PERFUSED RAT LUNG

**BY**

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# SUMMARY

The inhalation route is increasingly being considered as a viable option to deliver protein drugs into the body, but there has been few studies dealing with the safety of this strategy. The results of *in vitro* studies have shown that proteins, especially cationic proteins, can interfere with pulmonary surfactant and affect its surface tension lowering activity. If such an interaction also occurs *in vivo* it may lead to the inactivation of endogenous pulmonary surfactant and have profound adverse effect on the respiratory mechanics of the lung. To investigate this contention a suitable model which allows the inhalation mode of administration of proteins and the continuous monitoring of lung compliance and other parameters is needed.

The objectives of this study consequently were to (1) adapt the isolated perfused rat lung (IPL) to allow the administration of exogenous protein via the inhalation route into the alveoli, and (2) to use the adapted model to investigate the effect which inhaled cationic poly-peptides could have on lung function. It was hypothesised that such inhaled cationic peptides would interact with and inactivate the pulmonary surfactant leading to a decrease in lung compliance.

The lungs from adult Wistar rats were isolated and mounted in the IPL system. Three administration methods viz. aerosol administration, propellent driven administration and intra-tracheal instillation during positive pressure ventilation were considered. To evaluate the effectiveness of each method small volumes of fluorescein solutions were administered and the attained pulmonary distribution patterns assessed. To investigate

the effect of inhaled proteins different doses of the poly-peptides, poly-glutamate (poly-Glu MW 81,500), poly-lysine (poly-Lys MW 99,500) and poly- arginine (poly-Arg MW 92,000), were administered to the IPL using the intra-tracheal instillation method. The effects which these poly-peptides had on lung compliance, tidal volume and lung resistance, and post experimental lung weight were compared to that produced by saline.

Of the three administration methods intra-tracheal instillation with positive pressure ventilation resulted in an apparent good alveolar distribution and minimal effects on respiratory mechanics in the IPL. On the other hand the mist obtained with the aerosol nebulizer system was unsatisfactory and the lung distribution pattern of fluorescent dye obtained with the propellent driven system inconsistent and very limited. The administration of 150 µl of saline using the intra-tracheal instillation method produced acceptable changes in lung compliance (21.4% decrease), tidal volume (18% decrease) and lung resistance (4.2% decrease), 10 minutes after administration and this method was consequently used to administer the poly-peptides to the IPL. While the effects of the administration of 2.25 mg poly-Glu and 0.0225 mg each of poly-Lys and poly-Arg were not statistically different to that produced by saline administration, 0.225 mg doses of poly-Lys and poly-Arg produced significant 49.6% and 41.5% decreases in lung compliance at 6 minutes after administration, 51% and 46.2% decreases in tidal volume at 20 minutes and 47.2% and 32.2% increases in lung resistance at 20 minutes, respectively. At 2.25 mg doses of poly-Lys and poly-Arg the respective changes were 58.27% and 58.25% decreases in lung compliance at 2 minutes after administration, 39.2% and 46% decreases in tidal volume and 37.1% and 35.7% increases in lung

resistance, respectively. These higher doses also caused edema with significant increase in the ratios of lung weight to body mass.

From these results it is concluded that with the use of intra-tracheal instillation and short_duration positive pressure ventilation the IPL can be a suitable model to investigate the effects which inhaled poly-peptides might have on lung function. Also high molecular weight cationic poly-peptides, in contrast to anionic poly-peptides, administered into the alveoli of the IPL cause a significant dose-dependent deterioration in lung mechanics. Furthermore, the inhalation of high doses of high molecular weight cationic poly-peptides can, probably as a result of the impairment of pulmonary surfactant, lead to edema in the IPL. Collectively, these findings strongly suggest the administration of protein drugs (as pharmaceuticals), and especially those having a strong cationic nature, via the inhaled route, *in vivo* may also be expected to produce profound adverse effects on lung function. This should be the topic of urgent future investigation.

# CHAPTER 1

## INTRODUCTION

Pulmonary surfactant is a mixture of phospholipids and associated proteins secreted by type ll pneumocytes. Its main function is to reduce the surface tension at the air/water interface in the alveoli and small airways, thus maintaining morphologic stabilization of alveoli and preventing lung collapse at the end-expiration period. Several of the constituents of pulmonary surfactant appear to collectively contribute to this main function. The major component of pulmonary surfactant is the disaturated lipid, dipalmitoylphosphatidylcholine (DPPC) and this zwitterionic lipid appears to be responsible for the formation of a rigid film which dramatically reduces the surface tension of the alveolar air-liquid interface upon exhalation. About 5-10% of the total lipids of pulmonary surfactant is phosphatidylglycerol (PG), an anionic lipid, which possibly aids in the respreading of the DPPC over the expanding interface during inhalation. The protein components of surfactant (e.g. SP-A) influence the structure and properties of surfactant lipids. It is likely that the biological activity of pulmonary surfactant can be adversely affected if the functions of some of its components are impaired due to an interaction with some substances (such as proteins) which enter the alveoli.

Indeed, it is known that the surface activity of the pulmonary surfactant can be inhibited by such proteins as plasma, serum proteins and fibrinogen, etc. which leaks from the circulation into the alveolar air space. There is also strong evidence that

suggests that cationic poly-peptides can interact with the pulmonary surfactant and thereby dramatically increase the surface tension in the surfactant mono-layer. In addition, the cationic liposomes have been shown to inhibit the function of lung surfactant. These discoveries suggest that the proteins and cationic substances may interact with surfactant components leading to inhibition of the surfactant function of lowering surface tension at the air/liquid interface. It is very likely that the basis for this effect is an electrostatic interaction between the cationic substances introduced and the surfactant components, especially the anionic lipids. Eventually, the outcomes of such a substance-induced impairment of the surfactant functions could be a reduction in lung compliance, lung edema and alveolar collapse as occurs typically in adult respiratory distress syndrome (ARDS). Inhaled cationic proteins may therefore be particularly important candidates that could affect the normal function of pulmonary surfactant.

More and more new protein drugs are being developed and tested in clinical trials, but there are few convenient and efficient ways to get such drugs into the body. The inhalation route is consequently increasingly being considered as an effective approach to deliver these new drugs into the body. With the increase use of the inhalation route of administration, however, the risk that these protein drugs may interfere with surfactant function is, of course, correspondingly increased. Of particular concern is the possible occurrence of an interaction and/or formation of complex between inhaled cationic proteins and pulmonary surfactant, which will render the surfactant inactive and/or give rise to adverse effects in the lung. If the surfactant is inactivated changes in lung compliance and gas exchange will be expected. Presently, it is however largely

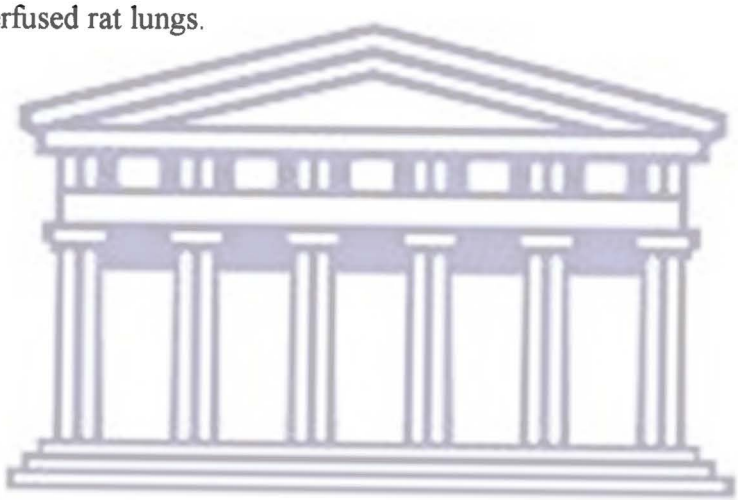
unknown whether cationic proteins could affect the activity of surfactant *in vivo*, though the cationic protein-induced inhibition of surfactant activity has been shown *in vitro*. For the latter studies high molecular weight polymers were used as models for the proteins. To investigate whether such an interaction might also occur *in vivo*, and to gain some perspective of its implications, the availability of a suitable experimental model is however crucial.

As a minimum, the model to investigate the effect which inhaled cationic proteins might have on pulmonary surfactant function must enable both drug delivery that can reach the alveolar sac, and the continuous monitoring of changes in respiratory mechanics. The isolated perfused lung model (IPL) could meet these criteria. If appropriately set up it has the unique advantages of allowing both the continuous monitoring of lung mechanics (e.g lung compliance, airway resistance, etc.) and the inhaled administration of drugs. It is also in a special position between *in vivo* whole animals and *in vitro* cultured cell experiments and allows the measurement of whole lung function devoid of influences of the central nervous system and other factors from the animal body. Further, both positive pressure or negative pressure ventilation can be used in the IPL to administer a cationic protein solution via the inhalation route in such a way that the protein reach the alveolar sac where the lung surfactant is located.

With the above as background, it was therefore hypothesised that exogenous cationic proteins administered to the IPL via the inhalation route will interact with the pulmonary surfactant, influence the latter's function in the lung and lead to a decrease in lung compliance. It was further accepted that high molecular mass cationic polymers

could serve as suitable models of cationic proteins to investigate this hypothesis.

The objectives of this study were consequently to (1) adapt the IPL to administer cationic polymers via the inhalation route so that it reaches the alveoli and (2) to use the adapted model to investigate the effect which such inhaled cationic polymers could have on lung functions. As examples of polymers poly-L- arginine (MW 92,000), poly-L-lysine (MW 99,500) and poly-L-glutamic acid (MW 81,500) were investigated in isolated perfused rat lungs.



# CHAPTER 2

## LITERATURE BACKGROUND

### 2.1 SYNTHESIS, COMPOSITION AND FUNCTIONS OF PULMONARY SURFACTANT

#### 2.1.1 THE SYNTHESIS OF THE SURFACTANT

Pulmonary surfactant is a mixture of many molecular species, mainly phospholipids and specific proteins (1), which is located in the alveolar sac. It is synthesized, stored and secreted by alveolar type II cells. In fact, its major component, phosphatidylcholine, is synthesized in the endoplasmic reticulum of the type II cell (Fig.1) (2), transferred to lamellar bodies which are, in turn, secreted into the alveolus. When lamellar bodies are exocytosed with loss of the limiting membranes from type ll cells, they are in the form of condensed, highly structured surfactant lipoprotein packages of about 1 μm average diameter (3). The tightly packed lamellar bodies then unravel to form tubular myelin structures as well as loose membranous arrays (4-6). Tubular myelin, a complex bi-layer structure, is considered to be the main source from which a surface-active mono-layer is formed (1). After surfactant is secreted into the alveolus, it may then be taken up by the type II cell or degraded by alveolar macrophages (7). All the components of pulmonary surfactant, including the four surfactant apoproteins, are synthesized in type II cells (8). Each component is expected to contribute to the overall functions of surfactant.

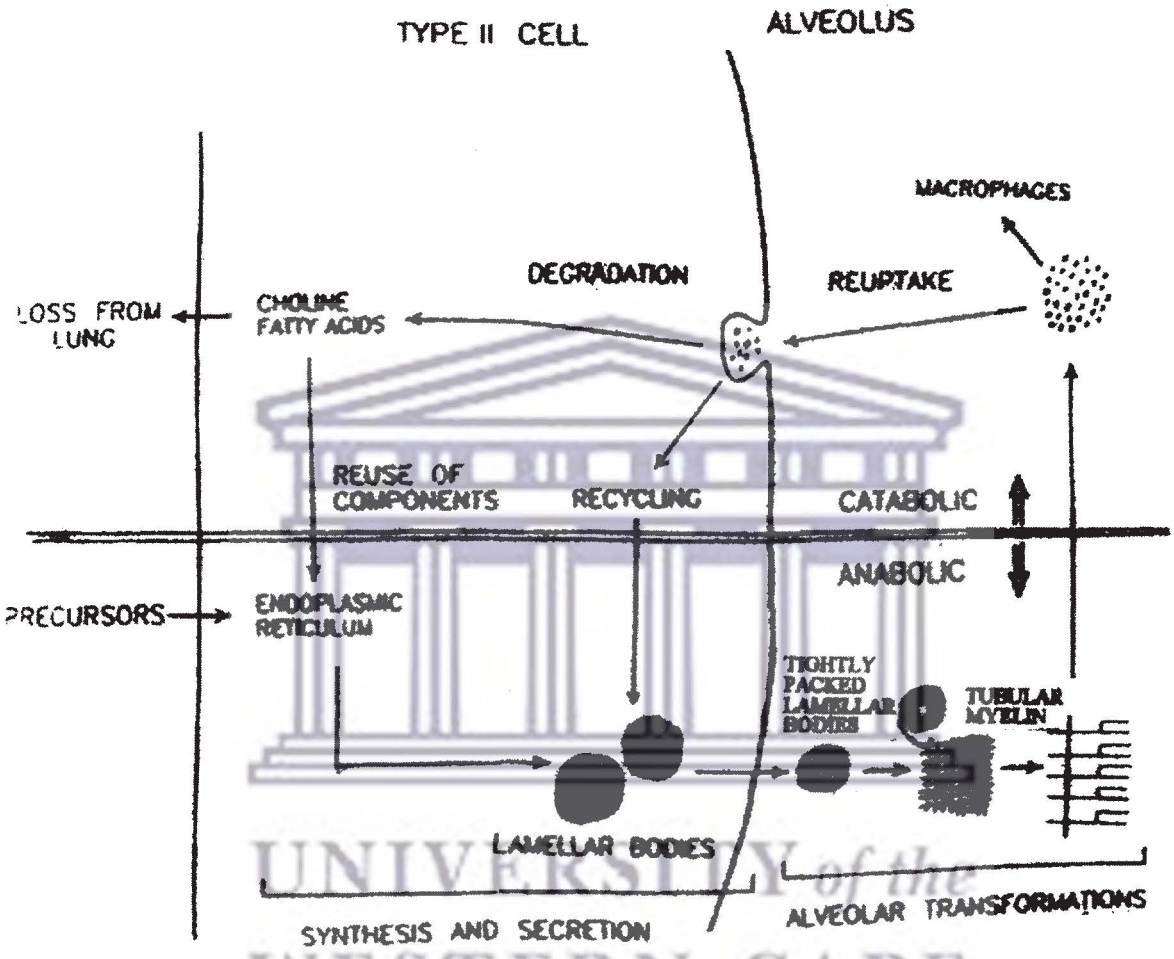





Figure 1 Diagram of the major pathways of surfactant phosphatidylcholine within the type II cell and the alveolus

## 2.1.2 THE COMPOSITION AND FUNCTIONS OF THE SURFACTANT LIPIDS

The compositions of the surfactant material obtained from several mammalian species are very similar (9). It contains 80-90% lipids by weight and more than 80% of the lipid are phospholipid (10). The major phospholipid components are phosphatidylcholine (PC) (60-70%); phosphatidylglycerol (PG) (5-10%); phosphatidylethanolamine (PE) (5-10%); and phosphatidylinositol (PI) and phosphatidylserine (PS) (PS+PI=3-6%) (11). The disaturated, zwitterionic lipid dipalmitoylphosphatidylcholine (DPPC) makes up about 70% to 75% of the PC fraction (12,13). Another important feature of the lung surfactant lipids (and ultimately their biophysical behaviour) concerns the class and levels of anionic phospholipids, most notably PG, PI, and PS, found in isolated surfactant. The result of several studies have shown the overall level of anionic compounds in the lavage and lamellar body to be essentially equivalent at 12% to 13% (see reference 14). The DPPC, as the most prevalent component of the surfactant, is responsible for the formation of a rigid mono-layer which dramatically reduces the surface tension of the interface upon exhalation (15,16) and which "splints" the alveoli open at small internal alveolar diameters (17). Anionic lipids such as the PG also play a role in the surface behaviour of pulmonary surfactant, possible by aiding in the respreading of the DPPC over the expanding interface during inhalation (15,18). Available evidence suggests that it may be better to consider the anionic lipids as a class, rather than PG in particular, when looking at a possible functional role for the anionic lipids in the activity of natural lung surfactant (14). It is clear that the surfactant lipids play an important role in the

surface activity of pulmonary function, but so do the surfactant proteins.

### 2.1.3 THE COMPOSITION AND FUNCTIONS OF THE SURFACTANT PROTEINS

The surfactant proteins (SP) include the four specific non-serum proteins SP-A, SP-B, SP-C and SP-D which comprise approximately 10% of the pulmonary surfactant by weight. SP-A and SP-D are water-soluble and collagen-like proteins that belong to the family of C-type lectins. SP-B and SP-C are small cationic hydrophobic proteins derived from a much larger precursor protein (8). SP-A, SP-B and SP-D have also all been detected in the gastrointestinal tract. SP-C, in contrast, appears to be a unique protein with extreme structural and stability properties and to exist exclusively in the lung (1). SP-A with monomeric molecular weight of 28,000-36,000 Daltons (Da) is rich in negatively charged amino acids (19). The monomer weights of SP-B, SP-C and SP-D are 9,000 Da, 4,000 Da and 43,000 Da, respectively (8). SP-A, the major surfactant protein, is thought to promote formation of tubular myelin-like structures from newly secreted phospholipids (20), and, in cooperation with the other surfactant proteins, to enhance the adsorption of phospholipids to the air-liquid interface (21). SP-A also regulates the metabolic path ways of secretion (22) and uptake (23) of surfactant between the type ll pneumocyte and alveolar space. SP-B and SP-C act to accelerate the adsorption and spreading of surfactant phospholipids at the air-liquid interface (24,25). SP-B also interacts with SP-A and calcium ions to promote tubular myelin formation (20). Further, SP-A and SP-D are involved in innate host-defence functions (1).

As a whole, the surfactant complex is thought to stabilize alveoli by reducing surface tension at the alveolar air-liquid interface (26-28), .and thereby prevent the lungs from collapsing at the end of expiration. It may have the additional functions of preventing lung edema and fluid transudation into alveoli (29,30), aiding the removal of foreign particles from airways, and assisting digestion of bacteria (31,32). It is thus very likely that if certain substances, such as proteins for instance, interfere with some of the components of surfactant, the latter's biological activity could be irreversibly impaired.

## 2.2 EFFECT OF PROTEIN AND POLY-CATION ON PULMONARY SURFACTANT ACTIVITY

As early as 1965, it was reported that pulmonary surfactant can be inhibited by plasma or serum contamination (33). In studies of proteins interfering with the surface activity of surfactant, the ability of various agents, such as fibrinogen, human serum, albumin, etc., to inhibit the activity of surfactant have been successively assessed. The results showed that the strongest inhibitory action to pulmonary surfactant activity was exerted by fibrinogen, followed by human serum, a 55,000 Da serum protein and globulin while the weakest inhibitor was albumin (34,35). These effects were dose dependent (35). Since these agents are all components of blood, a more frequent and serious problem might be the severe impairment of surfactant activity that could arise if the leakage of circulatory proteins into the alveolar air spaces occurs. Indeed in some pathological conditions (36), particularly fibrinogen and its products might react with surfactant and destroy the latter's ability to lower surface tension (37).

When circulatory proteins leak into the alveoli, it can lead to pulmonary oedema. This is a common clinical feature of adult respiratory distress syndrome (ARDS) where the major problem appears to be that of increased permeability of the capillary endothelium and excessive microvascular leakage of circulatory fluid and proteins resulting in alveolar oedema which prevents gas exchange in some lung units that are still perfused (36,38-40). The circulatory proteins leaking into the alveoli can also interfere with and impair the function of pulmonary surfactant and this may play an important role in this disease (34). Indeed, the results of various calculations (41) and experiments (42) support the idea that a high surface tension in the lungs, which would occur as a consequence of inadequately functioning surfactant, can lead to pulmonary edema.

Based on these observations which suggest that plasma proteins can inhibit pulmonary surfactant activity, it is postulated that other exogenous proteins delivered into the lung also have the potential to adversely affect the biological function of pulmonary surfactant (43). Support for this view is derived from the fact that cationic poly-peptides have been shown, *in vitro*, to influence the surface properties of pure films of anionic lipids at the air/water interface (44,45) and to alter the physical properties of anionic lipid vesicle systems (46-48). Since this potential for proteins to interact with and adversely affect pulmonary surfactant activity exists, and needs to be avoided, an understanding of the mechanism for this interaction may be quite important.

## 2.3 MECHANISM FOR INTERACTION BETWEEN PROTEIN OR POLY-PEPTIDES AND PULMONARY SURFACTANT

So far there have been few studies done on the mechanism of the interaction between protein or poly-cations and pulmonary surfactant. In general, surfactant function requires modest concentration of mono-or divalent cations, but may be inhibited by high concentration of di-or trivalent cations (49). Divalent cations may be involved in the formation of cation-phosphatidylcholine bridges, phospholipid-protein interactions, and conformational changes of the surfactant proteins (50). Our interest is however centred on the mechanism of interaction between surfactant and larger molecules.

As far as the interaction between protein and surfactant interaction is concerned some investigators speculate that the proteins effectively compete with the surfactant for space on the surface (34,51) or that they leave the surface and carry surfactant lipid along with them (34). Others think that lipid-protein complexes are formed in the surface and that these decrease the effectiveness of the lipid to reduce surface tension (34). From the results of their experiments, Seeger et al (52) concluded that the fibrin monomers interfered by forming a complex with one or more of the surfactant components (52).



In recent years some workers have also focussed their attention on the study of the effect that poly-cations have on surfactant activity. The report from Bummer et al (43) clearly showed that the presence of cationic poly-amino acids in the sub-phase of a buffer ( 0.15 M NaCl, 0.01 M phosphate, pH 7) inhibited the ability of the surfactant film to rapidly attain the plateau surface pressure considered necessary to maintain alveolar morphology. They also showed that the cation-surfactant interactions were dependent upon the concentration and molecular weights of the poly-amino acids (43),

the higher molecular weight polymers perhaps having a higher affinity for the surfactant-covered interface, in agreement with the cooperativity model proposed by Kim et al (53). More importantly, this experiment revealed that electrostatic interactions between the anionic lipids of the surfactant and cationic substances can be a mechanism for influencing the surface activity of surfactant.

As a basis for the electrostatic interactions between cationic substances and the anionic lipids of surfactant, the anionic phospholipids might be assumed to supply the putative binding sites for the poly-cation in the surfactant film (16,18). The cationic residues of the interfering substance then probably reside in the vicinity of the ionized head groups of the anionic lipids (45,54). The results of one study of cationic amino acid structures clearly showed that the side chains of such compounds are positively charged under physiological conditions, are strongly polar and usually found on the exterior surfaces, where they can be hydrated by the surrounding aqueous environment (55). These characteristics of cationic amino acids would make it easy for them to be attracted to the anionic lipids in the interface and be inserted into the phospholipid film (43). The electrostatic interaction mechanism is further supported by the results of another study which revealed that pulmonary surfactant may have a potential to compete with cationic lipid-DNA complexes for cell-surface binding sites or endocytosis (intake) and vice versa (56).

The surfactant-to-inactivator ratio is another factor influencing pulmonary surfactant surface activity. When the amount of surfactant increases, the anionic components of surfactant are generally also increased. Under these circumstances the effect of any

potential cationic inactivator to adversely affect such surfactant relatively declines (49). However, from an opposite perspective, surfactant activity can be decreased with the increase of concentration of any cationic substance. Duncan et al (56) have in fact proved that surfactant activity could be inhibited by cationic lipids if a certain ratio of surfactant to cationic substances are exceeded.

In summary, there is ample evidence supporting the view that cationic substances may, as a result of electrostatic interaction with the anionic phospholipids of surfactant, adversely affect the activity of pulmonary surfactant. Cationic proteins would be important candidates for such an effect. To do so these proteins must however first reach the alveolar spaces where the functional surfactant is found.

## 2.4 THE ADMINISTRATION OF PROTEIN DRUGS VIA THE LUNG

More and more new peptide and protein drugs e.g. recombinant human interferon-α (57), recombinant human granulocyte colony-stimulating factor (rhG-CSF)(58), etc. are being developed and the pulmonary route for their administration is being increasingly used in many research programs. Because extensive pre-systemic elimination renders peptide and protein drugs ineffective when they are administered orally, the systemic delivery of macromolecular drugs has generally been limited to the parenteral route. The lung with its relatively low extracellular enzymic activity (59) and extremely large and thin absorptive mucosal membrane however offers a particularly attractive non-oral route as an alternative for the absorption of such drugs (60). Tremendous efforts have thus been made to explore pulmonary drug delivery for the

systemic delivery of peptide-protein drugs, but only limited success has to date been achieved. Essentially only small peptides can be absorbed via this non-invasive route (61) and it has been reported that peptides and protein drugs such as insulin (59), interferon-alpha (57) and recombinant human granulocyte colony-stimulating factor (58), etc. can be efficiently absorbed via the pulmonary route. However, the systemic bioavailability of macromolecular drugs such as rhG-CSF after pulmonary delivery is still generally low (58). In this respect the existence of the barrier to airway-blood transport of high molecular weight compounds is worthy of consideration.

The structure of the barrier to peptide drug absorption in the lung consists of the surfactant, alveolar epithelium, capillary endothelium, basement membranes, and interstitial space. The pulmonary surfactant is the first layer of this barrier which could prevent protein drugs from reaching the alveolar wall. Once protein drugs mix with surfactant, they may coat surfactant vesicles (62) and/or specific molecular interactions between proteins and surfactant (e.g. charge interaction ) may alter the conformation of the surfactant lipid and protein (63). Such interactions could affect protein drug penetration to the alveolar wall. Beyond this first layer of barrier lies the alveolar epithelium with its smaller pores (0,8 to 1 nm) compared to that of the endothelium (4.0 to 8.0 nm) which also will also affect the permeability of different molecular weight solutes (64). The point to note therefore is that it is very difficult for proteins to pass through this complex combined barrier and, as a result, a large proportion of a dose of protein drug administered via the pulmonary route will be available for action in the alveoli. This then raises the concern whether such unabsorbed proteins or peptides (esp. cationic peptides) administered via the pulmonary route can adversely

affect surfactant activity (as discussed previously).

However, at the present stage, all of the studies involved in investigating the effects of proteins or cationic poly-peptides on pulmonary surfactant activity have made use of *in vitro* methods such as the Wilhelmy balance, Wilhelmy plate and maximum bubble pressure in glass capillary methods. One is however not able to directly assess the possible impairment of lung function and its possible clinical implications using such *in vitro* experimental models. A more appropriate experimental model would be crucial for such an investigation.

## 2.5 MODEL TO STUDY EFFECT OF CATIONIC PROTEIN OR POLY-PEPTIDES ON PULMONARY SURFACTANT ACTIVITY

The measurement of surface tension or surface pressure is widely used to investigate the activity of pulmonary surfactant (43,63,65) and has been used to investigate the effect of cationic protein or poly-peptides on the surface activity of pulmonary surfactant (43). From a measurement of surface pressure applied to the surfactant mono-layer, the value of surface tension can be obtained (66). In general, there are three methods available to measure the changes in surface pressure when studying surface tension lowering activity of pulmonary surfactant. They are the Wilhelmy Balance, the Pulsating Bubble Surfactometer and the Captive Bubble Surfactometer methods (35,52,63,65,67). Generally speaking, these methods have the advantages of being simple procedures with high precision and low cost. They are however *in vitro* experiments and parameters of many aspects of lung mechanics cannot be derived

directly from them.

To investigate the influence of surfactant on lung mechanics *in vivo* models can be considered. Such *in vivo* models have particularly been used in studies of surfactant replacement in ARDS and to identify the efficacy of the surfactant treatment on improving lung mechanics. In essence these models depend on either the initial deficiency of surfactant or the removal of the functional surfactant or the destruction of the activity of surfactant. Example of a model based on the first principle is the use of preterm rabbits (e.g. at 27 days gestational age) (68). Examples involving the removal of surfactant include the lung lavage adult guinea-pig (69) and rabbit (70) models. As examples of the third method, the destruction of surfactant activity with intratracheal administered xanthine oxidase (71) in guinea-pigs or intravenous injection of anti-lung serum in guinea-pigs can be considered (72). In each of the above models, surfactant replacement has been shown to significantly improve lung mechanics. There are however several disadvantages associated with these in *vivo* models such as influence of other organ systems on the lung, difficulties of continuous and simultaneous monitoring of many aspects of lung physiology in small animals, etc.



The IPL model, on the other hand, may offer solutions to such problems as well as several advantages for this particular investigation. As a transitional model from *in vitro* experiment to intact animal, the isolated perfused lung represents a system much less complicated than the whole animal while preserving most of the integrity of the organ. It stands in between *in vivo* experiments with whole animal and *in vitro* experiments with cultured cells (73). The *in vivo* experiments may provide good

evidence that a certain substance affects the lung, while only the testing of this substance in the IPL allows the definitive assessment of its impact on the lung. This is so because perfused lungs can allow the continuous and simultaneous monitoring of many aspects of lung physiology (e.g. lung resistance, dynamic compliance, pulmonary vascular resistance, edema formation and gas exchange, etc.), which is, as yet, not possible *in vivo* in small laboratory animals (73). Unlike experiments with cell cultures, the function of the intact organ would be demonstrated in the perfused lung model. For example, the endotoxin-induced release of thromboxane can occur in pulmonary tissue *in vivo* (74) and in the perfused lung (75), but not in lung slices (74) or in isolated lung cells (76). On the other hand, the IPL also offers the opportunity of investigating the administration of multiple agents by two different routes and in different physical forms. For instance, the inhalation and lung circulation routes in the IPL model can be used simultaneously with agents of different physical forms such as aerosol and solution, and multiple samples can easily and frequently be obtained from the perfusate (73).



Like other types of experimental models the IPL also has its limitations. It is only suitable for studies of short duration since the lung mechanics deteriorate with time. Other limitations are the absence of nervous regulation and lymph drainage (73).

Despite these disadvantages the IPL model offers a direct and practical measurement of the lung function parameters that would be needed to evaluate the activity of pulmonary surfactant. The IPL should therefore be an ideal model to investigate the effect that exogenous cationic proteins may have on the biophysical activity of

pulmonary surfactant. If inhaled cationic proteins adversely influence pulmonary surfactant activity, the effect could be reflected in changes in parameters of respiratory mechanics such as lung compliance, tidal volume, etc. A primary objective of this investigation is therefore to adapt the IPL for such an investigation.

# CHAPTER 3

## PLAN OF WORK

The primary objective of this study was to assess the potential of inhaled cationic proteins to adversely affect lung function. The realization of this objective required:

(a) the establishment and the application of an appropriate experimental model, such as the isolated perfused rat lung model (IPL), and

(b) the use of this model to investigate the effect of inhaled cationic proteins on respiratory mechanics (e.g. lung compliance, etc.) in the IPL model.

### 3.1 THE ESTABLISHMENT AND APPLICATION OF THE ISOLATED PERFUSED RAT LUNG MODEL



Firstly, the model needed for the study preferably had to consist of the lung of a small animal, it had to be amenable to easy adaptation for drug administration via inhalation and allow the continuous monitoring of various parameters of lung function. It seemed that the general IPL model which had been used in the previous studies (77,78,79) in the Department of Pharmacology, UWC would, with minor changes, also be appropriate for use in this study. While both rat and guinea pig lungs could be used, the rat lung was chosen because it was cheaper, had been used more often in the IPL, was more robust and sturdy during surgery and was easier to control and keep viable

in the IPL system.

Another important consideration was finding a suitable method for administering the cationic proteins via the inhalation route in the selected model. Aerosolized administration, propellant driven administration of a small bolus dose and tracheal instillation combined with positive pressure ventilation were all viable options in the IPL. From a pilot study it was however apparent that the first two administration methods, i.e. aerosolized administration and propellant driven administration of a small bolus dose, would not be appropriate for this study. Firstly, the density of mist generated by the nebulizer and observed visually was too thin and light for both low and high concentrations of the high molecular weight protein solutions (e.g. MW 92,000, etc). Secondly, the pulmonary distributions of the protein solutions administered by propellant driven device, into the airway and alveoli was rather limited and mainly restricted to the large airways. It was then decided to try the last option. At first the necessary accessories for administration (i.e. a suitably adapted syringe and needle and holding device), had to be designed, manufactured and tested. It was further decided to assess the efficacy of the administration method on the basis of two factors, viz (1) the effective pulmonary distribution of the administered protein solutions and (2) the load or volume of solution the lung could endure without significant alteration in lung function. To qualitatively assess the pulmonary distribution pattern, various volumes of solutions containing a flourescent dye was to be administered to the IPL using the designed set-up and the distribution pattern of the dye in the lung viewed and photographed under UV light. Along with fluorescein the administered test solutions were to contain saline, the anionic poly-peptides, or the

highest or lowest doses of the two cationic poly-peptides, so that the influence of differences in charge, molecular weight and concentration on the distribution pattern could be ascertained. To determine the appropriate volume that could be used, the reduction in lung compliance induced by the different volumes was to be used as deciding parameter.

The selected volume should not by itself cause a decrease in lung compliance of greater than 25%. And the IPL system and mode of administration would be considered suitable if at least 3 reproducible experiments could be performed. The pilot study showed that a volume of 150 µl and an administration period of 6 minutes under positive pressure ventilation constituted the optimum conditions to provide effective drug distribution.

Another key requirement for success in this project was that the model should permit the continuous monitoring of several parameters of respiratory mechanics. The IPL consequently had to be set up such that, in addition to the perfusion parameters (e.g. perfusion pressure, etc), the key ventilation parameters of airflow, transpulmonary pressure and tidal volume could also continuously be measured or calculated. To accomplish this a data capture system comprising the Pulmodyn™ computer program and a personal computer (PC) was to be used. This system would also allow the real time calculation of several derived parameters such as lung compliance and resistance, as well as permit the storage of the data for later retrieval and analysis.

Once the IPL had been set up to allow the satisfactory administration of drug solution

and continuous monitoring of lung function, it was to be used to evaluate the effect of the inhaled cationic proteins.

## 3.2 INVESTIGATION OF THE EFFECT OF CATIONIC PROTEINS ON LUNG FUNCTION

The aim of this part of the investigation was to ascertain whether cationic proteins, possibly via interaction with pulmonary surfactant, could adversely affect lung function when administered via inhalation. To achieve this goal several issues had to be addressed:

(a) Models for the cationic proteins. Based on literature evidence (43), it was expected that only high molecular weight polymers may affect the lung function. Since the low molecular weight cationic polypeptide (MW 3970) at a dose of 2.25 mg in 150 µl saline did not induce any visual significant change of lung mechanics in an initial study, it was not further used in this study and it was decided to use high molecular mass polymers of cationic amino acids to serve as models of cationic proteins. Thus the 2 cationic poly-peptides poly-L-arginine (poly-Arg MW 92,000) and poly-L-lysine (poly-Lys MW 99,500) were considered because of their high molecular weight and because they had previously been shown to reduce surface tension lowering activity at air/water interface *in vitro* (43). As control the anionic poly-peptide Poly-L-Glutamic acid (poly-glutamate MW 81,500) was selected. The neutral poly-peptide, poly-L-leucine (poly-Leu MW 78,400) was also initially considered as control,

but was difficult to solubilize and was therefore eventually excluded.

(b) Dose dependency of poly-peptide effect. It was also of interest to determine whether there would be a dose-dependency in the effect of the poly-peptides and therefore it was decided to determine the effects of 3 different doses of each poly-peptide.

(c) Experimental protocol and outcome parameter. Once the lungs were installed in the IPL system four different stages were envisaged for each experiment; viz. a pre-equilibration period, an equilibration period, an administration period and a post-administration period. The primary outcome parameter to be used for the assessment of the effect of the inhaled poly-peptides was the change in lung compliance after poly-peptide administration relative to the pre-administration compliance. Because edema arose in the initial experiments with poly-Arg, the occurrence of lung edema as judged by an increase in the ratios of lung weight to body mass was identified as another parameter to monitor. In addition, several other parameters were to be monitored to mainly verify the viability of the preparation during each experiment. Finally, to test the hypothesis that cationic proteins will cause a reduction in lung function, the effect that the cationic poly-peptides would have on lung compliance was to be compared with that caused by the anionic poly-peptide and saline.

# CHAPTER 4

# MATERIALS AND METHODS

## 4.1 MATERIALS, EQUIPMENT, ACCESSORIES AND ANIMALS

The following reagents, proteins, equipment, accessories and animals were used in the isolated perfused lung preparation, the evaluation of the distribution of the protein solutions in the lung, the subsequent experiments to investigate the effects of the proteins on the pulmonary surfactant activity, and for the data collection and analysis.

### 4.1.1 REAGENTS AND PROTEINS

Sodium chloride, potassium chloride, calcium chloride 2-hydrate, potassium dihydrogen phosphate, magnesium chloride 6-hydrate, sodium hydrogen carbonate, glucose (all analytical grade, from *Holpro Analytics (PTY) Ltd., Johannesburg,RSA*); Urethane, poly-L-glutamic acid sodium salt (ave MW 81,500), poly-L-arginine hydrochloride (ave MW 92,000), poly-L-lysine hydrobromide, (ave MW 3970 and 99,500), bovine serum albumin (fraction V) (*Sigma-Aldrich South Africa, Vorna Valley, RSA*); Fluorescein sodium salt ( *Saarchem (PTY) Ltd., Muldersdrift, RSA*); Carbon dioxide gas (*Fedgas (PTY) Ltd., Parow Valley, RSA*)

### 4.1.2 EQUIPMENT AND ACCESSORIES

Tracheal cannula, pulmonary artery cannula, left atrium cannula (*manufactured by Instrument Workshop, University of the Western Cape, Bellville, RSA*); Digital pH meter, model PHM82 (*Radiometer A/S, Copenhagen, Denmark*); Chart recorder, model R-03 (*Rikadenki Kogyo Co., Ltd, Tokyo, Japan*); Computer, type 386, (*SAMPO Corporation, Taiwan*); Small operating table (*Bioscience Company, Kent, U.K .);* Electronic weighing balance, model GA110, (*Scientific & Precision Equipment (PTY) Ltd. Cape Town, RSA*); Ultra-violet cabinet, model 0-70, (*Ultra-violet Products, INC, San Gabriel, CA. U.S.A.*); Roller pump, model MS-4 REGLO/8-100, PLUGSYS modular central electronics system type 603, Ventilation Control Module (VCM) type 681, Timer Counter Module (TCM) type 868, External Input Modules (EIM) type 673/2&3, Analog Digital Converter (ADC) type 663, Validyne Pressure transducer, model DP45-24, Differential pressure transducer, model SP 2040D, HSE DC Bridge amplifier, along with a perspex artificial thorax chamber, ventilation pressure limiter, pressure equilibration vessel, perfusate reservoir and pneumotachometer (all as per IPL model, type 829), and the Pulmodyn™ software (*HUGO SACHS ELEKTRONIK (HSE), March-Hugstetten, German*); Vacuum Pressure gauge, model 7769 (*Control Instrument Products, Johannesburg, RSA*); Thermostated Circulation bath, model 02 pH623 (*Heto Pty Ltd. Denmark*); SP Validyne Differential Pressure transducer, model P/N0004-150 (*UTAH Medical Products,USA*); Universal Pressure Transducer Readout, model SC1001, *(Gould Statham Company, Oxnard, CA. USA*); Carrier Demodulator model CD12 ( *Validyne Engineering Corp Northridge, CA.USA*); Ultrasonic nebulizer, modl NE-UO6 (*OMRON Tateisi Electronics, Co., Japan* ); Autohaler Placebo Demonstration Unit (*Three M Pharmaceuticals (Pty) Ltd, Johannesburg, RSA*); UVP Gel documentation

System comprising the White/UV Transilluminator with Camera, UVP model GDS 7500; Short hand UV lamp, model UVL-56; Image Store Computer, UVP model 5000 and Sony Graphic Printer, model UP-890 CE, all from the local agent (*Syngene P O Box Roosevelt Park 2129, Johannesburg, RSA).* Canon[R] camera with lens 35-105 mm, model EOS-3, (*Canon Photo Company, Japan*).

### 4.1.3 EXPERIMENTAL ANIMALS

The male Wistar rats of body mass 280-370 g used in this study were obtained from *Provincial Animal Centre, Kuilsriver, RSA.* They were fed the rat food purchased from *Epol (Pty) Ltd., Johannesburg, RSA* and kept before experiments in well ventilated animal room on 12 hour light/12 hour darkness cycle as per protocol approved by the Animal Ethics Subcommittee of the UWC Research Committee.

## 4.2 EXPERIMENTAL METHODS



### 4.2.1 PREPARATIONS OF THE SOLUTIONS AND PERFUSION MEDIUM

The following solutions were used in this investigation and routinely prepared as detailed below.

The perfusion medium used consisted of 500 ml of Krebs-Hanseleit buffer that also contained 2% bovine serum albumin (BSA) and 0.1% glucose. The composition of the

Krebs-Hanseleit was as follows:

| | | | |
|---|---|---|---|
| NaCl | 118 mM | KCl | 4.75 mM |
| $CaCl_2$ | 2.54 mM | $KH_2PO_4$ | 119 mM |
| $MgSO_4$ | 1.19 mM | $NaHCO_3$ | 25 mM |

To prepare this medium two litres of a stock solution with the above composition except for sodium bicarbonate component were prepared in distilled water and refrigerated. Prior to the experiment, sodium bicarbonate (1.05 g) and glucose (0.5 g) were dissolved in 500 ml of the stock solution. Thereafter 10 g of bovine serum albumin was added to the solution without stirring. After the BSA was dissolved completely, the medium was filtered and then poured into the water-jacketed glass reservoir maintained at 37°C. Before the start of the experiment the pH of the medium was adjusted to between 7.3 - 7.4 by bubbling through $CO_2$ gas.



Each of the poly-peptide (i.e. poly-arginine, poly-lysine and poly-glutamic acid) solutions used was freshly prepared in 3 different doses (2.25 mg/150µl , 0.225 mg/150µl and 0.0225 mg/150µl ) using the following method:

(a) 2.25 mg poly-peptide/150µl solution was prepared by dissolving 5 mg of poly-peptide powder in 0.3 ml of physiological saline,

(b) 0.1 ml of the 2.25 mg poly-peptide solution was diluted with 0.9 ml of saline to give a 0.225 mg/150µl solution and

(c) 0.1 ml of 0.225 mg poly-peptide solution was diluted with 0.9 ml of saline to give a 0.0225 mg/150µl solution.

To prepare the fluorescent solutions (0.5%) 1.5 mg of fluorescein sodium was dissolved in 0.3 ml of saline or the different solutions of each of poly-peptides.

### 4.2.2 THE ISOLATED PERFUSED LUNG SYSTEM

The isolated perfused lung (IPL) system used in this study was essentially a modification of that used by Uhlig et al (73) and can be divided into four sections viz. : (1) negative-pressure chamber with lung; (2) perfusion apparatus; (3) ventilation system and (4) data capture system. A schematic diagram of the developed isolated lung system is shown in Figure 2.

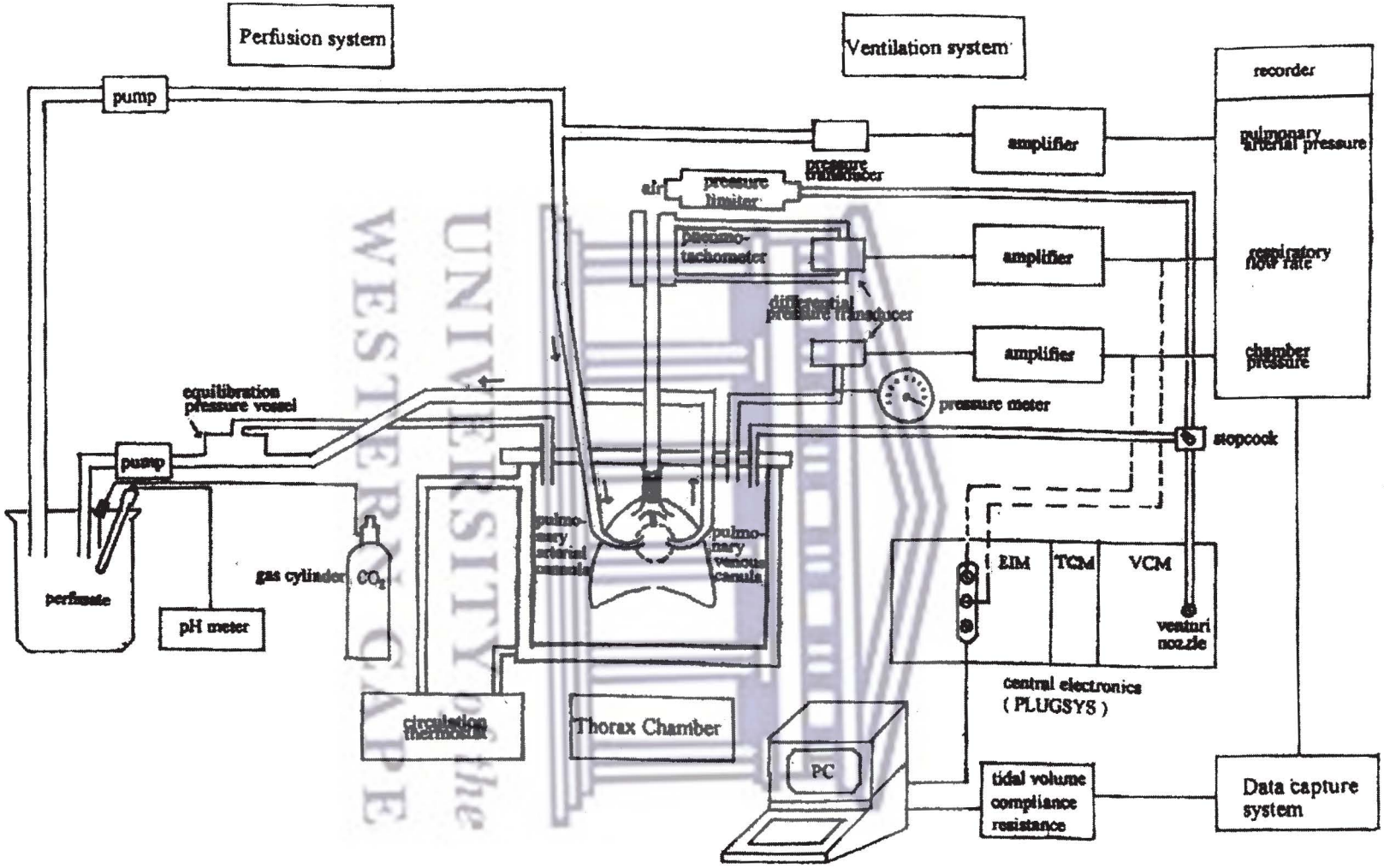



Figure 2 Diagram of the isolated perfused lung system

#### 4.2.2.1 THE NEGATIVE-PRESSURE CHAMBER WITH LUNG

In this system the lung was placed in a water-jacketed artificial glass thorax (Fig.3) fitted with a plexiglass lid. The latter had a central opening (i.e. organ holder), to which a pneumotachometer was connected, and two side holes through which 2 glass tubes penetrated. The lung was connected to the organ holder via the tracheal cannula, while the pulmonary artery was connected via a cannula to one of the 2 glass tubes. Perfusate was pumped into the lung via this pulmonary arterial cannula. The other glass tube was connected to a pulmonary venous cannula which was placed into the left ventricle for perfusate discharge. The lung chamber also had the following additional connections: one for the negative-pressure venturi nozzle; another linked to a chamber pressure transducer for measuring trans-pulmonary pressure (i.e. chamber pressure) and two other connections for the circulation of thermostated water (37°C). A small amount of water in the bottom of the lung chamber served to humidify the air. Finally, the drain was normally blocked by a plastic stopper.

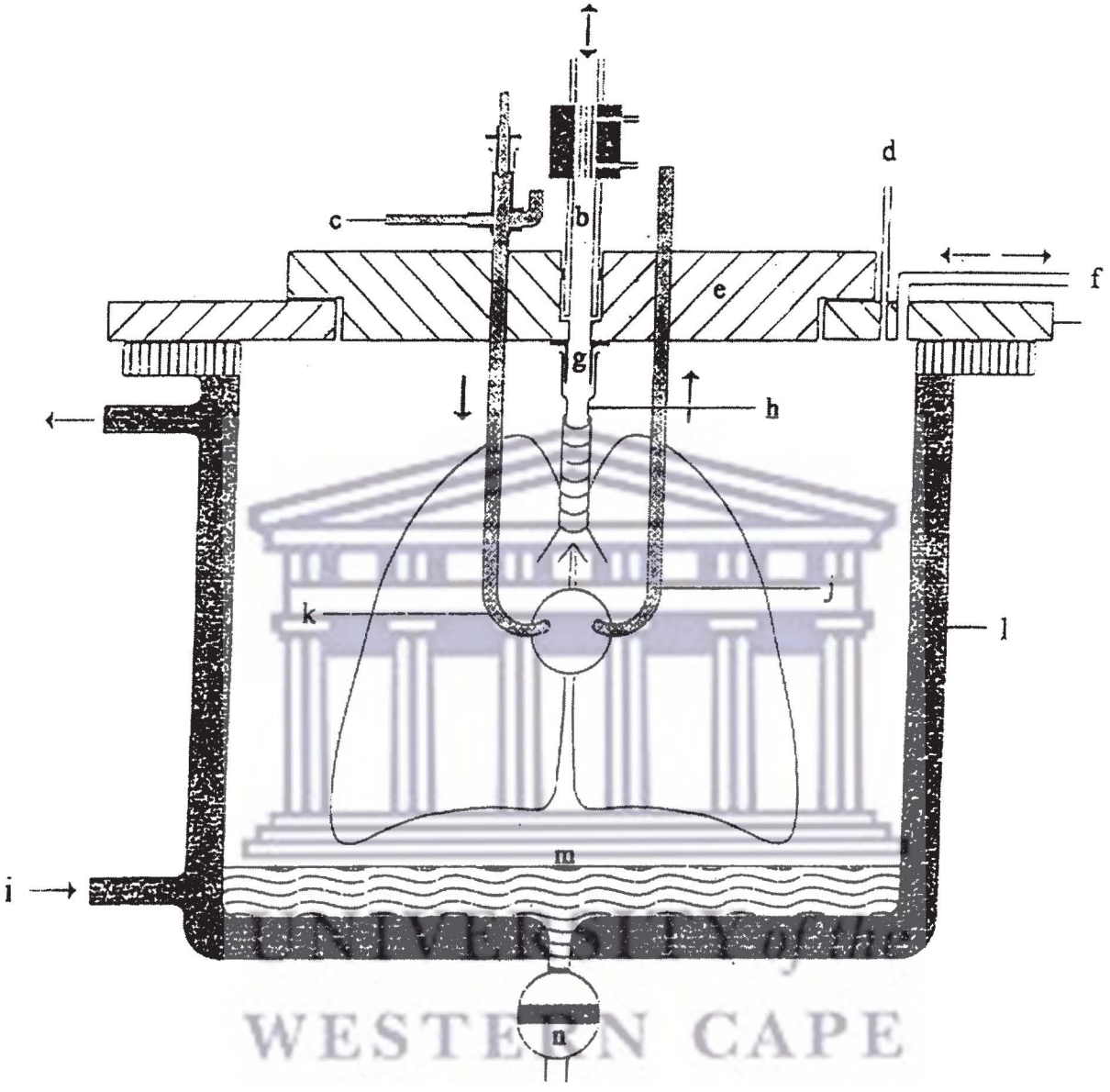





Figure 3 Diagram of the artificial thorax. (a) airflow; (b) pneumotachometer; (c) arterial inflow; (d) to chamber pressure transducer; (e) plexglass lid; (f) to venturi nozzle; (g) organ holder; (h) tracheal cannula; (i) inlet for the thermostated water; (j) pulmonary venous cannula; (k) pulmonary aetrial cannula; (l) water-jacheted aetificial thorax chamber; (m) water; (n) drain.

#### 4.2.2.2 PERFUSION APPARATUS

To supply the lung with the required nutrients necessary to maintain its working condition in the chamber, a roller pump was used to continuously drive perfusate (at 37°C) from a 500 ml water-jacketed reservoir through a short perfusion line to and through the lung vasculature. The perfusate entered the lung via the pulmonary artery and left it via the left ventricle back to the reservoir. Before returning to the reservoir the perfusate passed through a pressure equilibration vessel which was located at the level of the left atrium and was responsible for keeping the transmural pressure constant. The pulmonary arterial pressure (i.e. perfusion pressure) was continuously measured with a pressure transducer which was connected via thin tubing to the pulmonary arterial cannula. In preliminary experiments the flow rates at different pump speeds were determined by collecting the effluent over time. A table of pump rates vs perfusate flow rate was then drawn up and fixed pumps speeds were subsequently used in the experiments to realise the desired flow rates. During the experiment the pH of the perfusate in the reservoir was also monitored with a pH meter and it was maintained at 7.3 to 7.4 by periodically bubbling $CO_2$ gas through the solution.



#### 4.2.2.3 VENTILATION SYSTEM

The ventilation system needed to maintain the respiratory function of the lung consisted of several parts which were coordinated through a central electronics modular system, the PLUGSYS™. The main component of the system was the ventilation control module (VCM) which was used to generate fluctuating negative

pressures inside the chamber (via a venturi nozzle) or positive pressure ventilation (via a pressure limiter linked to the trachea). Between the lung or chamber and the VCM a stopcock was inserted to facilitate the change over from positive-pressure to negative-pressure ventilation during the experiment. The respiratory rate and the level of the end-expiratory and end-inspiratory pressure was controlled by the VCM settings. The frequency of deep breaths ("sighs") were adjusted through the Time Counter Module (TCM) of the PLUGSYS™. To measure the airflow passing through the lung a pneumotachometer was connected to the trachea via the central opening in the perspex lid. The signal from the pneumotachometer was passed via a differential pressure transducer (model SP 2040D), amplifier and external input module ( EIM ) in the PLUGSYS™ electronics unit to a computer. The respiratory volume was derived by the computer program (Pulmodyn™) from the flow signal. To measure the trans-pulmonary pressure the two arms of a differential pressure transducer (Validyne model DP45-24) was connected to the inside and outside of the chamber and the signal similarly amplified and passed on to the computer. A continuous visible indication of the chamber pressure was also obtained via a pressure meter connected to the chamber. Finally, normal air was used (via VCM) to ventilate the lung.



#### 4.2.2.4 DATA CAPTURE SYSTEM

To evaluate the respiratory function of the lung in the IPL, parameters of respiratory mechanics such as tidal volume ($V_T$), lung resistance ($R_L$) and dynamic compliance ($C_L$) were monitored. The signal for these parameters were fed into a computer via the PLUGSYS™. An analogue-digital (A/D) card and the Pulmodyn™ computer

software was used for data collection, manipulation, real time display and storage. At the end of the experiment a hard copy of the data were printed. In addition, the primary measured values of airflow rate, trans-pulmonary pressure and pulmonary arterial pressure was also monitored on a chart recorder (see appendix 7.1).

### 4.2.3 SURGICAL PREPARATION OF THE ISOLATED PERFUSED LUNG

The rat (280-370 g) was anaesthetized with urethane at a dose of 75 mg/kg injected intra-peritoneally. When the optimum depth of anaesthesia, as determined by testing for absence of the pain reflex (e.g. by clamping a front paw of the rat using an artery clamp), was achieved the anaesthetized animal was placed on the operating table in the ventral position. It was fixed to the table by tying its paws with elastic bands. Using a pair of scissors, the skin was incised ventrally in the median line from the upper abdomen to the neck and then separated from the muscles of the thoracic wall. The trachea in the upper cervical region was dissected taking care not to cut off the carotids on both sides of the trachea. A thread was drawn through underneath the trachea and looped loosely. A curved forceps was placed underneath the trachea to make it stretched. A small cut was then made in the trachea with a pair of small curved scissors, the tracheal cannula inserted and tied in place with the thread. Thereafter the cannula was linked to the organ holder of the chamber to which the pressure limiter was mounted beforehand. The ventilation control module (VCM) was immediately switched on and the positive pressure ventilation at 2 - 10 $cmH_2O$ started with 80 breaths/min with no sighs. The abdominal wall was incised from the upper abdomen to

manubrium sterni and the incision extended bilaterally along the rib bows of both sides. Then the two axillary arteries were cut to exsanguinate the animal. The diaphragm was carefully dissected from the thorax wall and the caudal mediastinal connective tissue cut through. The thorax was then opened by cutting through the left ribs close to the sternum and extending the cut to the trachea. Heparin sodium (2000 IU/kg) was injected into the right ventricle and the both halves of the thorax partially cut off so as to appropriately expose the lung. Further, most of the thymus was removed. Then, the apex of the heart was lifted up with a straight forceps and another small straight forceps inserted between the left auricle and the left ventricle of the heart behind the aorta and pulmonary artery. A thread was drawn through this space behind the two large vessels and loosely looped. The perfusion pump was briefly switched on to fill the perfusion line and ensure that the pulmonary artery cannula was free of bubbles. Using a pair of small curved scissors, the right ventricle was incised 5 mm from the start of the pulmonary trunk. The arterial cannula was inserted through the ventricular incision into the pulmonary artery and a ligature was drawn tight to simultaneously fix the cannula in the artery and close the aorta. Another ligature was placed loosely around the middle part of the heart. After raising the heart's apex with a forceps, a small transverse incision was made in the left ventricle close to the apex of the heart. The pulmonary venous cannula was then inserted through the ventricular incision and mitral valve into the left atrium. Care was taken to only apply the appropriate force needed to pass the cannula through the mitral valve without damaging the extremely thin pulmonary vein. The cannula was secured with the pre-looped thread but not too tightly. At this moment the roller pump was switched on and the lung perfused at the initial flow rate of 5 $ml.min^{-1}$. The inferior vena

thoracic aorta, the two vagal trunks, oesophagus and upper part of trachea were removed, and the lower part of trachea and lung dissected free by step by step cutting in the cranio-caudal direction. Finally, the isolated perfused lung was placed into the artificial thorax chamber and the lid secured in position.

After the lung was placed into the chamber the positive-pressure ventilation was stopped and negative-pressure ventilation started by simply switching the stopcock. The pressure in the chamber now fluctuated between an end-inspiratory pressure of -10 to -16 $cmH_2O$ and an end-expiratory pressure of -2 $cmH_2O$. Further, the perfusate flow rate was increased to 25 $ml.min^{-1}$ which resulted in a pulmonary arterial pressure of about 13 $cmH_2O$. Now, the pressure limiter was removed from the central opening of the chamber lid and the pneuotachometer installed in the opening to measure the airflow rate. Finally, the Timer Counter Module (TCM) of PLUGSYS™ electronics system was set to produce a hyperinflation (-16 $cmH_2O$) every 2 minutes. Under these conditions a tidal volume of 1.4 ml ±1.0 and lung compliance of 0.4-0.6 $ml.cmH_2O^{-1}$ was attained and shown on the PC monitor. The pH of the perfusate was maintained in the range of 7.3-7.4 by adding $CO_2$ gas and the lung allowed to equilibrate under these conditions. The lung was then observed and assessed if fit for use based on the following criteria, viz. whether, (1) all of the lobes of the lung were adequately expanded; (2) the lung colour changed from pink to white; (3) the air flow rate was not less than 10 $ml.sec^{-1}$; (4) the pulmonary artery pressure was between 10 $cmH_2O$ to 16 $cmH_2O$ ( average about 13 $cmH_2O$); (5) the trans-pulmonary pressure (TPP) was in range -2 to -10 $cmH_2O$ for the end-inspiration and -16 $cmH_2O$ for the maximum end-inspiration; (6) the tidal volume was in the range of 1.4 ml ±1.0; and (7) the lung

compliance was between 0.4 and 0.6 ml.cm$H_2O^{-1}$. If the lung did not meet any of these criteria, it was discarded. This pre-equilibration assessment lasted about 3 minutes and it took no more than 10 minutes from start of surgery to reach this stage. The lung was now ready for the experiment.

### 4.2.4 ADMINISTRATION METHODS

The following three methods of administration in the IPL were considered for use in this study: (a) aerosolized administration; (b) propellant driven administration of a small bolus dose and (c) tracheal instillation combined with positive pressure ventilation (Fig.4A, 4B, 4C). The method which proofed the easiest to use, permitted a relatively large volume of dose and gave the most consistent reproducible distribution to the alveoli was to be used for the administration of the poly-peptides.

#### 4.2.4.1 AEROSOLIZED ADMINISTRATION BY NEBULIZER



For this method a nebulizer was linked via thin silicone tubing with a 5 mm internal diameter (i.d) and a perspex connection to the central opening of the lid of the artificial thorax of the IPL (Fig.4A). The aerosol generated by the nebulizer flowed through the tubing and connection to the lung via the tracheal cannula. To determine the effectiveness of this administration system, 2 ml of saline or protein solution (2.25 mg in saline), with or without fluorescein, was placed in the small plastic bowl, aerosolized and the mist allowed to enter the lung. During such administration the lung was ventilated under negative pressure (see below).

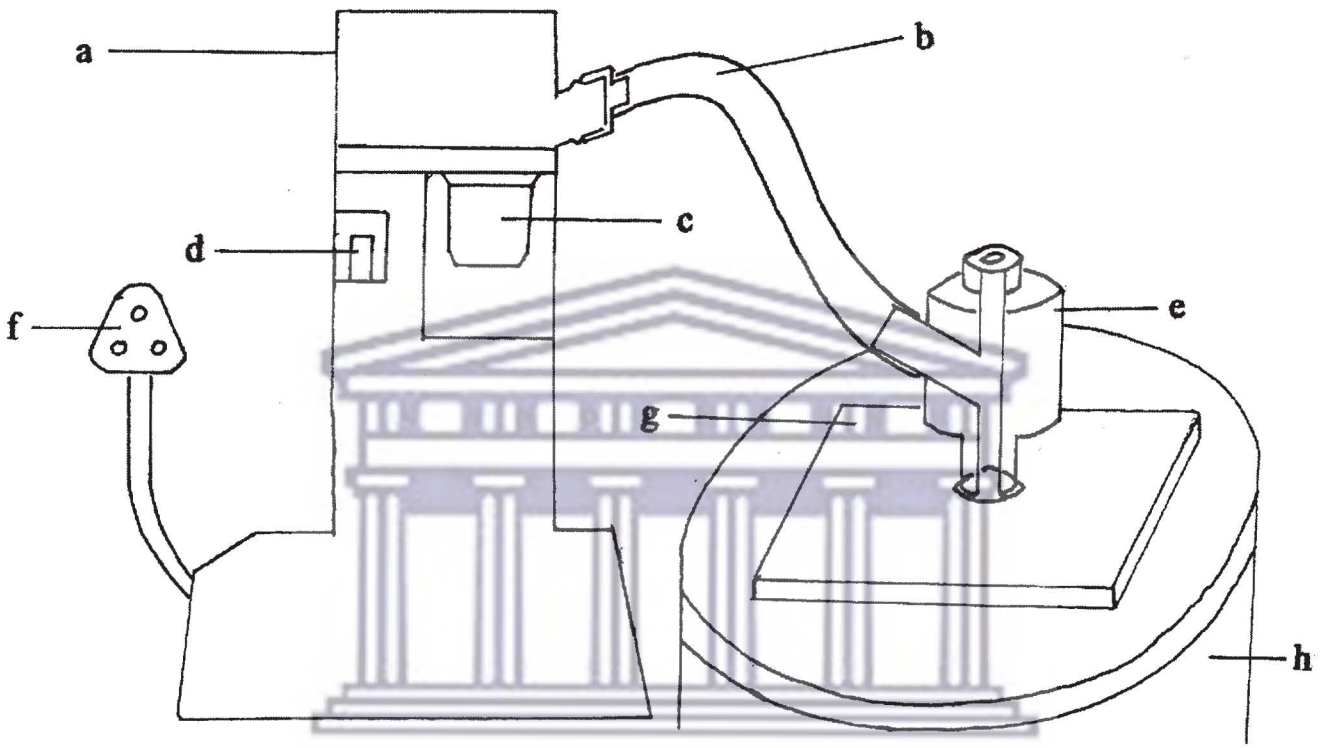





Figure 4A Diagram of the aerosol administration system. (a) nebulizer; (b) silicone tubing; (c) plastic bowl; (d) switch; (e) perspex connection; (f) power plug; (g) chamber lid; (h) artificial thorax.

#### 4.2.4.2 PROPELLENT DRIVEN ADMINISTRATION SYSTEM

This administration system consisted of a modified metered-dose inhaler (Autohaler™) which was linked through the sample-containing glass tube (1.8 mm i.d x 6.3 mm long), a 18 gauge (G) needle and the needle holding device (Fig.4B) to the lung. The Autohaler™ was modified so that it could be activated by a solenoid-driven plunger via an electronic trigger which was synchronised with the deep-inspiratory phase of the VCM. When the trigger was activated, the plunger opened the flap of the primed autohaler, releasing propellant, while at the same time the VCM facilitated a deep inspiration of the lung (i.e. caused a decrease in negative pressure in the chamber). To administer the drug solution, a small amount (eg. 150 µl) of solution was placed in the glass tube which was then inserted into the device. When the inhaler was triggered, the placebo propellant from the inhaler drove the sample into the lung while the latter took a deep breath. To test the effectiveness of this device various volumes (50 to 200 µl) of solutions containing fluorescein was administered and the lung examined for fluorescein distribution afterwards (see below).

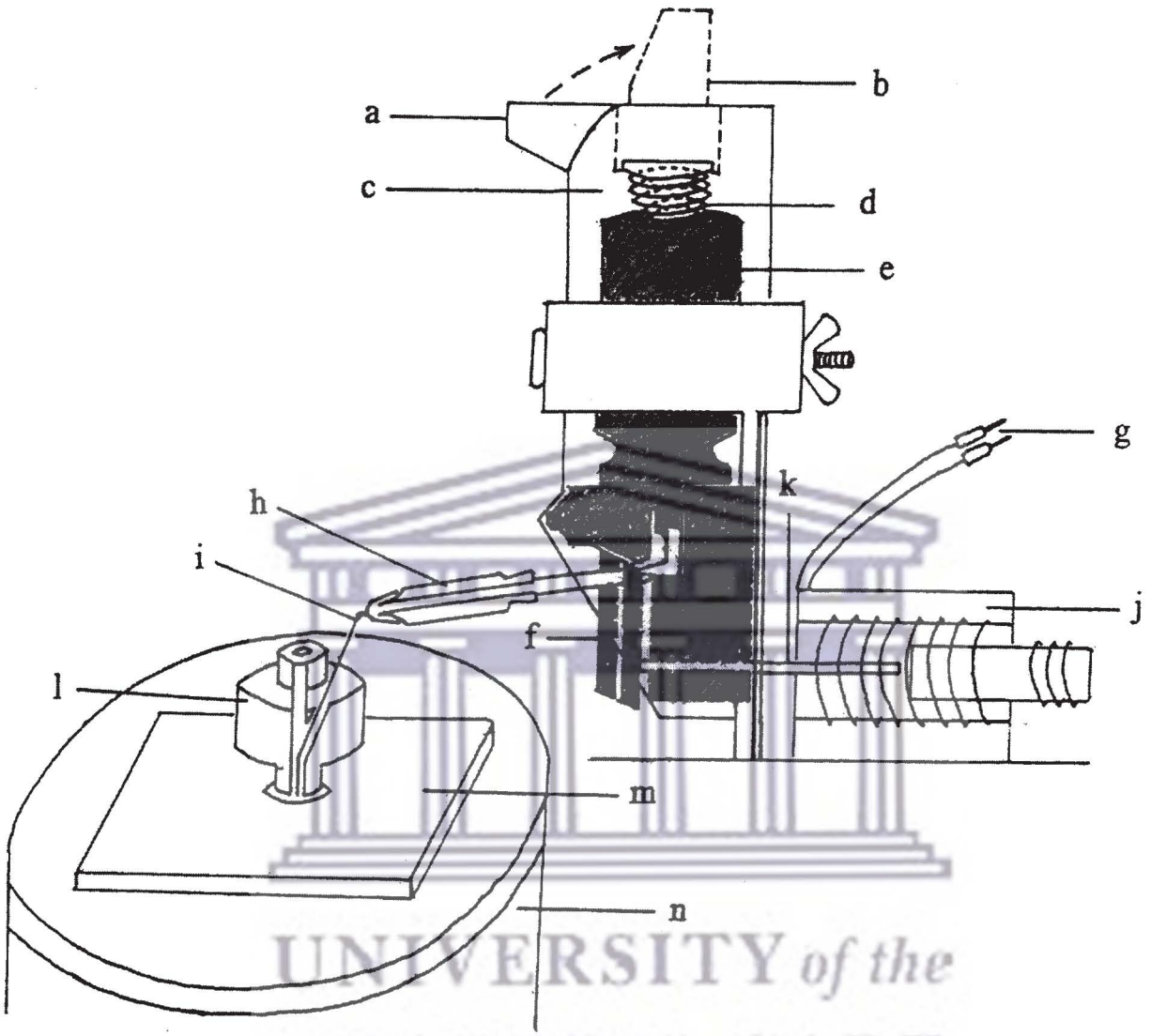





Figure 4B Diagram of the propellent driven administration system. (a) lever; (b) lever ( in the loading position ); (c) aerobec™ autohaler™ outside body; (d) spring; (e) autohaler pressured vial filled with placebo propellent; (f) flap; (g) to electronic trigger on VCM; (h) sample-containing glass tube; (i) needle; (j) solenoid-driven plunger; (k) piston; (l) needle holding device; (m) chamber lid; (n) artificial thorax.

#### 4.2.4.3 TRACHEAL INSTILLATION WITH POSITIVE PRESSURE VENTILATION

This administration system consisted of two parts (Fig.4C), viz. ventilation head (pressure limiter) and the perspex needle holding device in which a 25 G 3 ½ inch needle attached to1 ml syringe was fixed. The ventilation head was linked, on the one side, to the perspex needle holding device with silicone tubing and, at the other end, to the VCM. It also contained an adjustment screw with which the positive pressure ventilation could be controlled. Small volumes (50 to 200 µl) of saline or protein solutions (with or without fluorescein) were administered from the 1 ml syringe while positive pressure ventilation of the lung was continued uninterrupted.

### 4.2.5 THE PROTOCOL FOR THE EXPERIMENTS

The experiment was divided into the following periods: an equilibration period, an administration period, a post-administration period and a lung disposal process period.



#### 4.2.5.1 EQUILIBRATION PERIOD

After the lung assessed as fit for use, it was maintained in the chamber for a further 10 minute equilibration period. During this time (and throughout the rest of the experiment) the pH of the perfusate was maintained in the range of 7.3 to 7.4 and the relevant parameters of respiratory mechanics of the lung monitored on the PC screen

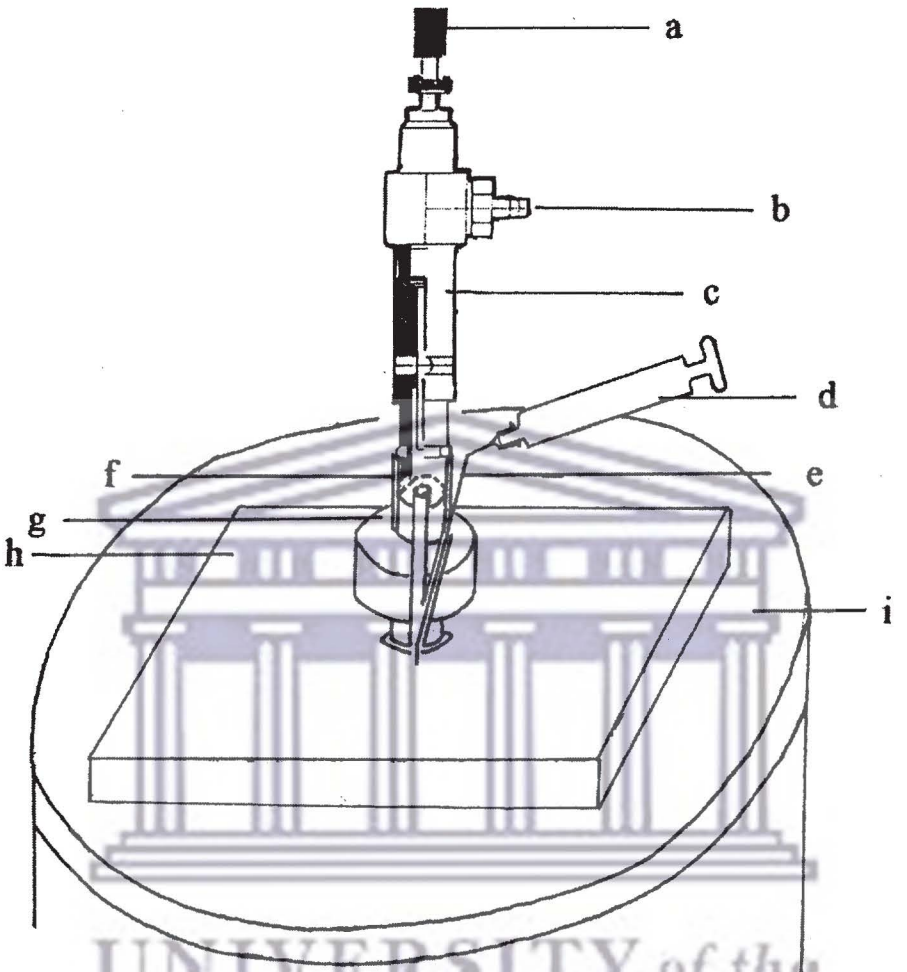





Figure 4C Diagram of the system for tracheal instillation with positive pressure ventilation. (a) respirator pressure control screw; (b) to VCM; (c) pressure limiter; (d) syringe; (e) needle; (f) silicone tubing connection; (g) needle holding device; (h) chamber lid; (i) artificial thorax

and chart recorder and stored on the PC. After 7 minutes, the perfusate flow was switched over from non-recirculating to recirculating mode. After 10 minutes, the present document file (document 1) was closed and the final preparation for peptide administration commenced.

#### 4.2.5.2 ADMINISTRATION PERIOD

Two series of experiments were performed. In the first series the various administration devices were tested as described under 4.2.2. In the second series of experiments where the effect of the poly-peptides were assessed, the pneumotachometer was removed from the chamber lid at the end of the equilibration period. The perspex administration device (Fig.4C) consisting of a 25 G 3 ½ inch needle attached to a 1 ml syringe containing 150 µl solution (saline in the control group or poly-peptides in the test groups) was inserted in place in the lid. Further, the pressure limiter was placed on top of the administration device. Once this had been done the time interval between minimum and maximum end inspiration was set to 30 seconds, the positive pressure ventilation started and the time noted. Thereafter, 50 µl of solution was administered into the lung after 10, 40 and 70 seconds. The time for administration of each 50 µl was kept within 10 seconds. After the administration of the solution the lung was maintained for a further 4 minutes and 40 seconds under the condition of positive pressure ventilation. During this period the pulmonary arterial pressure tended to rise, but did not rise above 20 $cmH_2O$. The entire administration period lasted 6 minutes and in this time a copy of document 1 was also printed (see appendix 4.1).

#### 4.2.5.3 POST-ADMINISTRATION PERIOD

Following the above period, the mode of ventilation was switched back to negative pressure ventilation, the administration device and pressure limiter removed from the chamber lid and the pneumotachometer reinstalled into the central opening of the chamber. Further, the time interval of hyperinflation was turned back to 2 minutes and the test document 2 on the Pulmodyn™ programme started. The changes in the parameters of lung mechanics were monitored and recorded continuously on the computer, but particularly noted at 5, 10, 15, 20, 25 and 30 minutes. After 30 minutes the experiment was terminated and document 2 printed (see appendix 4.2 and 4.3) and the chart recorder paper collected (see appendix 7.1).

#### 4.2.5.4 LUNG DISPOSAL PROCESS PERIOD

After both series of experiments (i.e. to test the administration devices and assess the effect of the poly-peptides) the lungs were assessed for edema and drug distribution as described below. To assess it for edema, the lung was taken from the chamber, placed in the horizontal position in a petri-dish and visually inspected for any obvious signs of edema eg. for the presence of translucent spots, change in size and whether foamy liquid emerged from the trachea. Thereafter the heart was dissected free and the lung with partial trachea weighed on an electronic balance. The results of these observations and the lung weights were recorded for the evaluation of the lung edema.

To assess the extent of pulmonary distribution of the solutions, the different lobes of

the lung were separated from each other by dissecting the connective tissue between the lobes. The lung with the separated lobes was then fixed with pins onto a small wooden plate (Fig.5) and viewed in a small UV cabinet under 366 nm wavelength UV light or with the UVP gel documentation system. The distribution pattern of the administered solutions were indicated by the fluorescence of the fluorescein contained in the solutions and this was photographed by a camera or generated by the gel documentation system.



Figure 5 Anatomy of rat lung

### 4.2.6 DATA ANALYSIS

Primarily 2 series of experiments were conducted. In the first series to ascertain the best administration technique to use, the 3 administration approaches were visually and qualitatively assessed on the basis of the pulmonary distribution patterns which they produced. In the second series the effect which the administered poly-peptides had on lung function was assessed on the basis of changes in lung compliance, tidal volume and lung resistance. In this series two sets of data were collected from each experimental lung. The first set was collected at 2, 6 and 10 minutes during the equilibration period and taken as baseline values. The second set of data were collected at 2, 6, 10, 20 and 30 minutes during the post-administration period. Each of these data points were the average of 12 continuous readings (for each parameter) taken over 2 minutes on the computer. The change in parameter values from the base line to post-administration period time points reflected the effect of the administered solutions. The magnitude of the change obtained after the administration of saline (control) and the various poly-peptide (test) solutions were compared. The mean data were analysed using Mann-Whitney U test and P values $< 0.05$ was considered significant. Level of significance ($\alpha$) was adjusted based on the number of comparisons of data points collected during the post-administration period. The Mann-Whitney $U$ test was used for the data analysis because it has good properties (asymptotic relative efficiency, etc.) for statistical analysis of small number of data, especially when the assumption of normality is in doubt. It is the most powerful or sensitive nonparametric alternative to the $t$-test for independent samples; in fact, in some instances it may even offer greater power to reject the null hypothesis than the $t$-test (80). According to the

advantages of *U* test suitability for investigation of small population, it was therefore chosen for data analysis in the present study.

# CHAPTER 5

## RESULTS

Essentially two series of experiments were conducted, viz. one series to establish the IPL model for this study and the other series to determine the effect of the cationic poly-peptides on lung function. The following results were obtained in the these 2 series of experiments.

### 5.1 ADAPTATION OF THE IPL MODEL

The primary adaptation to the IPL model required for this study was the establishment of an effective system to efficiently and reproducibly administer solutions via the inhalation route to the lung. And three methods were tried. The initial results obtained with the first method tried i.e. administration of aerosolized solutions were disappointing. The density of aerosolized mist generated from the nebulizer with the low concentrations of polypeptide solution was too thin and light as observed visually, and even worse at the higher concentrations. This method was therefore not considered viable for use in this investigation. The second method tried i.e. the propellent driven administration of a small bolus dose of solution, resulted in rather limited distribution (see Fig.6). Most of the solution ended up either in the large airways e.g. trachea or concentrated in a single or limited region. Also the cationic poly-peptide distribution seemed to differ from that obtained with the saline. Examples of the results attained with the third




Figure 6 Computer-generated photograph depicting pulmonary distribution of fluorescent dye after the administration of (A) saline and (B) cationic poly-lysine using the propellent-driven administration system. Bright white areas indicate presence of dye, dark area of the lung represent tissue without the probe.

method tried, i.e. tracheal instillation with positive pressure ventilation, are shown in Figures 7 to12. The distribution patterns obtained clearly indicated a wide distribution of the fluorescent dye to all parts of the lung, including the alveoli. The dye was distributed to all the lobes and there was no consistent pattern of distribution in any particular lobe, i.e. pattern varies from lung to lung. Also, the saline (Fig.7) and glutamic acid (Fig.8) solutions appeared to have a wider distribution than the cationic poly-peptides (Fig.9 to 12). However there was no apparent visible difference in the distributions of the two cationic poly-peptides, at both 2.25 mg and 0.0225 mg doses (Fig.9 to 12). Based on these results this method was used to administer the poly-peptides in the second series of experiments.



Figure 7 Photograph indicating pulmonary distribution of dye after the administration of saline. The intra-tracheal instillation method was used and the green colour indicates the presence of fluo-rescent dye, the blue areas lung tissue without fluorescent probe.



Figure 8 Photograph indicating pulmonary distribution of dye after the administration of 2.25 mg poly-glutamate solution. The intra-tracheal instillation method was used and the green colour indicates the presence of fluorescent dye, the blue areas lung tissue without fluorescent probe.



Figure 9 Photograph indicating pulmonary distribution of dye after the administration of 2.25 mg poly-lysine solution. The intra-tracheal instillation method was used and the light green colour indicates the presence of fluorescent dye, the blue areas lung tissue without fluorescent probe.



Figure 10 Photograph indicating pulmonary distribution of dye after the administration of 0.0225 mg poly-lysine solution. The intra-tracheal instillation method was used and the light green colour indicates the presence of fluorescent dye, the blue areas lung tissue without fluorescent probe.



Figure 11 Photograph indicating pulmonary distribution of dye after the administration of 2.25 mg poly-arginine solution. The intra-tracheal instillation method was used and the light green colour indicates the presence of fluorescent dye, the blue areas lung tissue without fluorescent probe.



Figure 12 Photograph indicating pulmonary distribution of dye after the administration of 0.0225 mg poly-arginine solution. The intra-tracheal instillation method was used and the green colour indicates the presence of fluorescent dye, the blue areas lung tissue without fluorescent probe.

In the addition to using an appropriate method of administration, the viability of the isolated perfused lung and the reproducibility of the measured respiratory parameters were also assessed to confirm the successful adaptation of the model. For these experiments 150 µl of saline was administered via tracheal instillation under positive pressure ventilation and the tidal volume, lung resistance and lung compliance monitored. These results are shown in Figures 13, 14, 15 and appendix 1.1, 2.1, 3.1 respectively. There was a 18.0% decrease in $V_T$ at 10 minutes after the period of saline administration. Thereafter the $V_T$ decreased at a rate of 0.0068 $ml.min^{-1}$ over the next 20 minutes (compared to a rate of 0.0058 $ml.min^{-1}$ which prevailed before the saline administration). On the other hand, there was a 4.2% increase in lung resistance at 10 minutes after the period of saline administration and a further increase at a rate of 0.0012 $cmH_2O.ml^{-1}.sec^{-1}.min^{-1}$ over the next 20 minutes (compared to a rate of 0.0004 $cmH_2O.ml^{-1}.sec^{-1}.min^{-1}$ prior to the saline administration). Finally, there was a 21.4% decrease in lung compliance at 10 minutes after the period of saline administration and a further decrease at a rate of 0.005 $ml.cmH_2O^{-1}.min^{-1}$ over the next 20 minutes (compared to a rate of 0.0042 $ml.cmH_2O^{-1}.min^{-1}$ prior to the saline administration ).



## 5.2 THE EFFECT OF POLY-PEPTIDES ON LUNG FUNCTION

The goal of the second series of experiments was to determine the effect which various poly-peptides had on lung function compared to the effect of saline.

### 5.2.1 EFFECT ON THE TIDAL VOLUME

The results depicting the effect which the various poly-peptides had on tidal volume is shown in Figure 13 and appendix 1.1, 1.2.

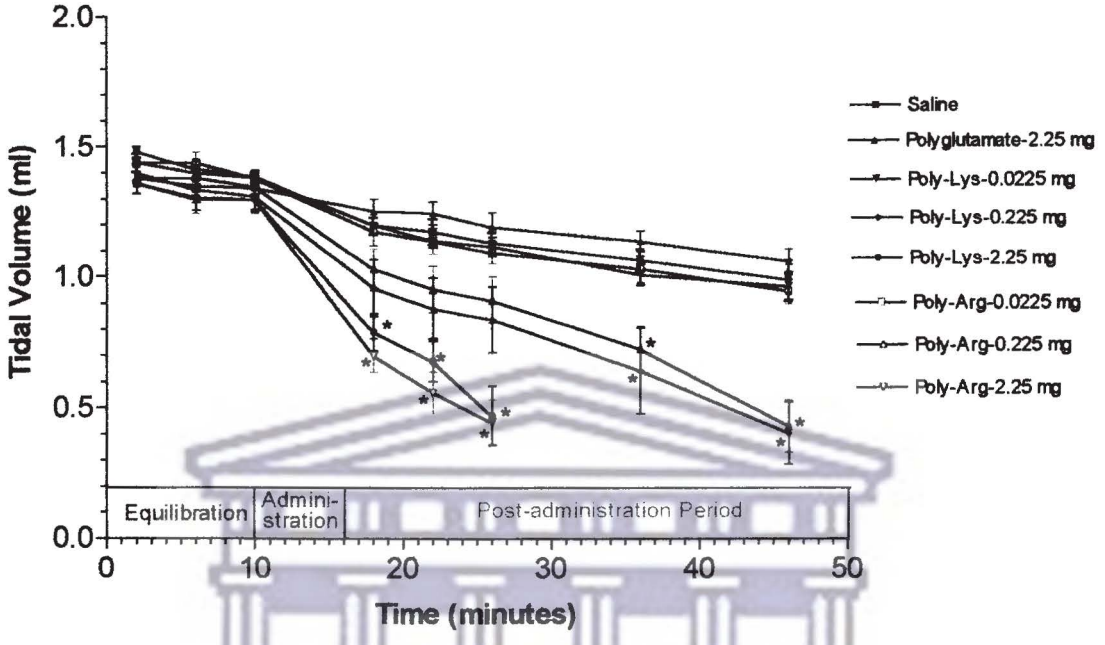



Figure 13 The effect of different poly-peptides on tidal volume of the lung in the IPL. Individual poly-peptides were administered by intratracheal instillation during positive pressure ventilation over 6 minutes (administration period). The mean ( and SE ) obtained for n = 5 lungs are shown and * indicates the data points significantly ($P < 0.05$) different from that of saline.

The anionic polypeptide (poly-glutamate, 2.25 mg) produced a 11.2% decrease in the tidal volume at 10 minutes after administration and decreased further at a rate of 0.007 $ml.min^{-1}$ over the next 20 minutes. The average $V_T$ at 2, 6, 10, 20 and 30 minutes was not statistically different from that obtained with saline. The low dose (i.e. 0.0225 mg) of the cationic poly-peptides, poly- Lys and poly-Arg decreased the $V_T$ at 10 minutes

by 19.3% and 20.6%, respectively, which was also not significantly different from that caused by saline. Neither were the values at later times. However the two higher concentrations (0.225 mg and 2.25 mg) of poly-Lys and poly-Arg produced significant decreases in $V_T$ at 20 and 2 minutes respectively after administration compared to that produced by saline. The 0.225 mg solutions of poly-Lys and poly-Arg produced decreases of 36.4% and 32.3% at 10 minutes and 51% and 46.2% at 20 minutes respectively. The 2.25 mg solutions induced decreases of 39.2% and 46% at 2 minutes and 63.7% and 65.5% at 10 minutes respectively. After 10 minutes, the respiratory condition of the lung deteriorated sharply and no valuable readings can be recorded on the computer.

### 5.2.2 EFFECT ON LUNG RESISTANCE

The results depicting the effect which the various poly-peptides had on lung resistance is shown in Figure 14 and appendix 2.1, 2.2. The anionic polypeptide (poly-glutamate, 2.25 mg) produced a small 1.8% increase in the lung resistance at 10 minutes after administration and increased further at a low rate of 0.0007 $cmH_2O.ml^{-1}.sec^{-1}.min^{-1}$ over the next 20 minutes. The average lung resistance at 2, 6, 10, 20 and 30 minutes was not statistically different from that obtained with saline. Similar results were obtained with the low dose (i.e. 0.0225 mg) of the cationic poly-peptides, poly-Lys and poly-Arg which caused increases in lung resistance of 6.2% and 6.8% respectively at 10 minutes. The two higher doses (0.225 mg and 2.25 mg) of poly-Lys and poly-Arg however produced significant increases in lung resistance at 20 and 2 minutes respectively after administration compared to that produced by saline. The 0.225 mg

solutions of poly-Lys and poly-Arg produced increases of 25.2% and 20.6% at 10 minutes, 47.2% and 32.2% at 20 minutes and increased even further over the next 10 minutes. Thirty minutes after administration the lung resistance had increased by a total of 77.1% and 106%, respectively. Finally, the 2.25 mg poly-Lys and 2.25 mg poly-Arg caused dramatic increases in lung resistance of 37.1% and 35.7% at 2 minutes and 84.0% and 78.4% at 10 minutes after administration, respectively.

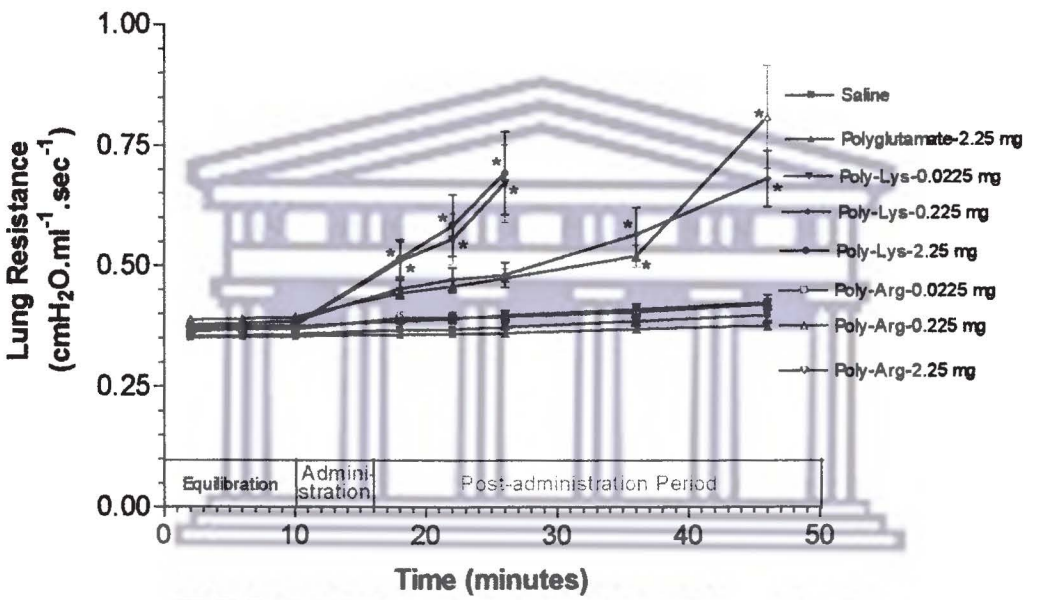





Figure 14 The effect of different poly-peptides on lung resistance in the IPL. Individual poly-peptides were administered by intra-tracheal instillation during positive pressure ventilation over 6 minutes (administration period). The mean ( and SE ) obtained for n = 5 lungs are shown and * indicates the data points significantly ($P < 0.05$) different from that of saline.

### 5.2.3 THE EFFECT ON LUNG COMPLIANCE

The results depicting the effect which the various poly-peptides had on lung compliance is shown in Figure 15 and appendix 3.1, 3.2.

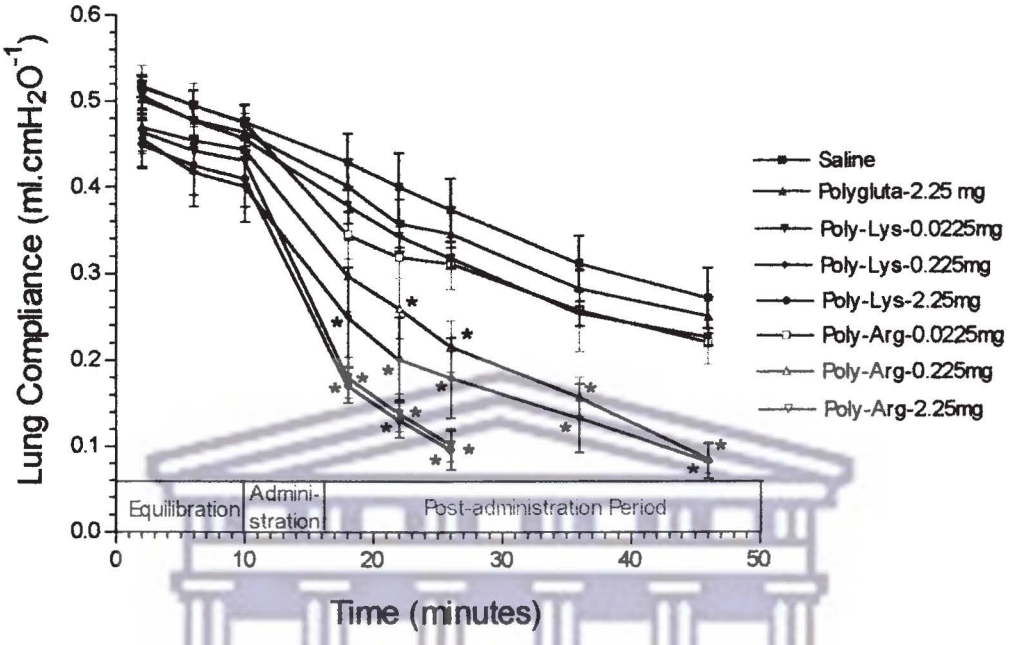



Figure 15 The effect of different poly-peptides on lung compliance in the IPL. Individual poly-peptides were administered by intra-tracheal instillation during positive pressure ventilation over 6 minutes (administration period). The mean (and SE) obtained for n = 5 lungs are shown and * indicates the data points significantly ($P < 0.05$) different from that of saline respectively.

The anionic polypeptide (poly-glutamate, 2.25mg) produced a 25.5% decrease in the lung compliance at 10 minutes after administration and decreased further at a rate of 0.0047 $ml.cmH_2O^{-1}.min^{-1}$ over the next 20 minutes. The average lung compliance at 10, 20 and 30 minutes was not statistically different from that obtained with saline. The low dose (i.e. 0.0225 mg) of the cationic poly-peptides, poly-Lys and poly-Arg

decreased the lung compliance at 10 minutes by 30.0% and 34.3%, respectively, which was also not significantly different from that caused by saline. Neither were the values at later times. However the two higher doses (0.225 mg and 2.25 mg) of poly-Lys and poly-Arg produced significant decreases in lung compliance at 6 and 2 minutes respectively after administration compared to that produced by saline, and even further decreases over the subsequent time. The 0.225 mg solutions of poly-Lys and poly-Arg produced decreases of 49.6% and 41.5% at 6 minutes respectively and the 2.25 mg solutions decreases of 58.27% and 58.25% at 2 minutes after administration, respectively.

### 5.2.4 EFFECT OF LUNG EDEMA

In this investigation the ratios of lung weights measured after the experiments to body mass were calculated to give an indication of edema formation. The results are given in Figure 16 and appendix 5. In the lungs to which saline was administered the average ratio of lung weight to body mass was 0.00596 +/- 0.000025 (n = 5). The administration of 2.25 mg poly-glutamate and 0.0225 mg poly-Lys and 0.0225 mg poly-Arg produced no significant change in their final ratios of lung weight to body mass. After the administration of 0.225 mg poly-Lys and 0.225 mg poly-Arg the mean ratios increased to 0.00874 +/- 0.000277 and 0.00864 +/- 0.000509 (n = 5), respectively, while the administration of 2.25 mg poly-Lys and 2.25 mg poly-Arg resulted in ratios of 0.0142 +/- 0.00086 and 0.015 +/- 0.000775, respectively. The ratios produced by the higher doses of cationic poly-peptides were significantly different from that found after saline administration, with $P < 0.05$ for the 0.225 mg

and the 2.25 mg doses. In the lungs exposed to the 0.225 mg concentration of cationic poly-peptides a small amount of the light yellow foamy liquid came out of the lung when it was placed in the horizontal position. In the lungs dosed with the highest concentration of cationic poly-peptide the same liquid actually overflowed from the trachea even while the lung was suspended vertically. No fluid emerged from the lungs exposed to the other doses and type of poly-peptide.

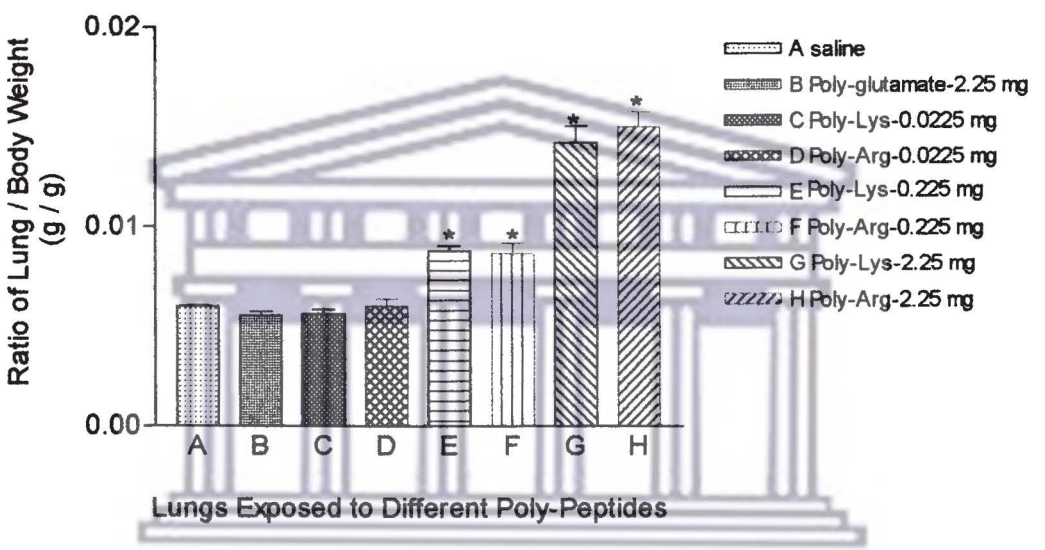





Figure 16 The effect of the different poly-peptide solutions on post-experimental lung weight. Histogram depicts the mean ratio of lung weight to body mass (g/g) (and SE ) achieved for n = 5 lungs each. * Indicates significant ($P < 0.05$) increase in the ratios compared to that of saline (A).

# CHAPTER 6

# DISCUSSION

## 6.1 SUITABILITY OF IPL

The first objective of the present study was to adapt the IPL model to study the effect of the inhaled cationic poly-peptides on the activity of pulmonary surfactant. Prior to this study, investigations of the interaction between poly-cations and surfactant mainly involved the measurement of the surface pressure or surface tension in the surfactant film and for this methods based on the Wilhelmy Balance, Maximum Bubble Pressure, Pulsating Bubble Surfactometer, etc were employed (35,65,67). Although these methods have the advantages of being simple procedures which offer precise readings at relatively low cost, they do not provide a direct measure of the effect which surfactant may have on lung function or the parameters of lung mechanics. For the latter, the IPL model had a distinct potential to be suitable for this study. As a transitional model in between the *in vitro* experiments with cultured cells and *in vivo* experiments with whole animal, the IPL represented not only a system much less complicated than the whole animal, but also one which preserved most of the integrity of the organ (73). It allowed the continuous and simultaneous monitoring of many aspects of lung mechanics (e.g. dynamic compliance, lung resistance, pulmonary vascular resistance, edema formation and gas exchange, etc.) which is, as yet, not readily possible *in vivo* in small laboratory animals, let alone in *in vitro* experiments. In addition, the IPL model also provided a feasible inhaled drug delivery route (i.e.

inhalation route) to the lung. However, the respiratory tract is a complex system of branching tubes of progressively decreasing size. This progressive reduction in size presents a severe challenge to drug delivery, since the inhaled drug particles are constantly having to change direction and, in moving through air of progressively decreasing velocity, their propensity to deposit increase dramatically (81). To realise the primary objective of this investigation, it was therefore critical to design or choose an effective administration method to drive small particles of the cationic proteins into the alveolar sac. Three methods of administration were consequently tested in this study.

The first method tried, viz. aerosol administration with the aid of a nebulizer, proved totally unsatisfactory. The protein in saline solutions simply did not nebulize well enough to make this method a realistic option for this study. For the second method an elaborate propellent driven device (Fig.4B) was constructed to drive 150 µl of solution through a thin needle into the lung at the critical point of deep inspiration. This method however also proofed unsatisfactory. The distribution of the solution into the lung was very limited and poorly reproducible (Fig.6). There may be several reasons for this. Perhaps the administration time (one or a few deep breaths) was too short and the subsequent negative pressure ventilation (at normal tidal volume) inadequate to promote the spread of the solution into the alveoli. The size of the particles produced with this device was also unknown and may have contributed to the unsatisfactory results attained. Also the force generated by the device briefly overinflated the lung, but did not appear to have a long-term effect on the lung. For this method to have been of use some additional modifications were therefore

obviously required, and while this was possible for future studies it was not considered for the present study. Finally, Byron and Niven (82), using a similar method, speculated that 65.9 +/- 4.8 % of their administered dose was deposited in the lung periphery, but gave no indication of where in the periphery. The visual data obtained in the present study however did not indicate such substantial and consistent distribution of solution to the alveoli. Since the latter was the critical requirement for this study, this method for administration of the polypeptide was therefore not considered further.

The third administration method tried, viz tracheal instillation of a bolus dose under positive pressure ventilation, proved satisfactory for the present study. It produced good and reproducible alveolar distribution of the solutions in the isolated perfused lung. The even and steady air flow generated by the positive pressure ventilation, at 10-16 cm $H_2O$, appeared to be an effective force to drive the small particles of solutions through the branching tubes into the alveolar sacs in the different lobes within the 6 minutes time of administration. It also did not appear to adversely affect the parameters of lung function. While the administration of saline caused changes in parameters of lung function, positive ventilation without administration of solution had no effect. Interestingly, the saline and glutamic acid solutions appeared to have a better distribution in the lungs than the cationic poly-peptide solutions. This difference may be due to a charge reaction between the poly-cations and the fluorescein sodium salt which should be absent when the latter is omitted. This issue however was not investigated further. Finally, the distribution patterns obtained in the present administration model was better than the uneven distribution of intra-tracheal administered suspensions which Brain et al (83) and Pritchard et al (84) had found in

*in vivo* models.

Together with the intra-tracheal mode of administration the IPL system appeared to be ideally suited for this study. he required parameters such as lung compliance and tidal volume that was needed to evaluate the effect on surfactant function could easily be monitored in the present system. For this investigation experimental conditions were set such that tidal volumes of 1.3 to1.5 ml, lung compliance of 0.4 to 0.6 ml.cm $H_2O^{-1}$ and lung resistance of 0.33 to 0.40 cm $H_2O.ml^{-1}.sec^{-1}$ were obtained in the system, similar to values used by some previous researchers (85,89). Under these conditions the lungs remained viable for the required time, but the saline administration had a distinct solution volume dependent effect on the lung function parameters. While the administration of 50 µl of saline did not affect any of the parameters significantly, 150 µl saline produced some changes in tidal volume, lung compliance and lung resistance immediately after administration. These changes were however within acceptable range and the 150 µl was considered an optimal volume for the administration of the poly-peptide solutions. As such then the adapted IPL model appeared suitable to investigate the effect of inhaled cationic poly-peptides on the function of pulmonary surfactant.



## 6.2 EFFECT OF INHALED POLY-PEPTIDES

Based on the hypothesis that the interaction of cationic poly-peptides with anionic components of surfactant *in vitro* (43) impairs the surface tension lowering activity of surfactant we postulated that such a reaction *in vivo* or in the IPL will result in

changes in the parameters of lung respiratory mechanics. Specifically, if this reaction prevailed, the administration of inhaled cationic poly-peptides should cause a decrease in lung compliance and tidal volume and an increase in lung resistance.

In this study we found that anionic poly-peptides had essentially no effect on respiratory mechanics in the IPL. High doses (2.25 mg) of poly-glutamate, relative to saline, caused only minor insignificant changes in tidal volume, lung resistance and lung compliance. From this it may be concluded that this type of poly-peptide most probably does not interfere with surfactant function (certainly not beyond a volume effect which was also seen with saline). Such a conclusion is in accordance with the findings from the *in vitro* study by Bummer et al (43) who found that poly-glutamate (average MW 100,000) did not affect the equilibrium surface pressure when injected under a pulmonary surfactant film.

On the other hand the cationic poly-peptides poly-Arg and poly-Lys significantly affected the respiratory mechanics of the IPL in a dose dependent manner. While the lowest dose (0.0225 mg) did not produce changes much greater than that seen with saline, the tidal volume and lung compliance were significantly decreased, and lung resistance significantly increased, at the higher 0.225 and 2.25 mg doses. In addition, the two poly-cations used in this study had similar high molecular weights to that used by Bummer et al (43), viz. poly-Arg (average MW 139,000) and poly-Lys (average MW 100,000), and was selected on the basis of the *in vitro* finding that the higher molecular weight polymers have a higher affinity for the surfactant-covered interface. Indeed, in the pilot study to the present investigation the administration of high doses

of low molecular weight poly-Lys (MW 3970) to the IPL did not produce any apparent change in lung respiratory mechanics and confirmed the appropriateness of this selection. Apart from the molecular weight, the type of poly-cation, i.e. whether poly-Lys or poly-Arg, did not appear to play a significant role with regard to the effect of the poly-peptide. This is also in agreement with the conclusion of Mosir and McLaughlin (48) who studied the interaction of basic arginine and lysine residues with membrane containing acidic lipids and found that these basic residues bound phosphatidylglycerol (PG) and phosphatidylserine (PS) equally well respectively.

Since Bummer et al (43) had shown in their *in vitro* study that these cationic poly-peptides interact with surfactant, it may be concluded from the present findings that these high molecular weight cationic polymers also affected lung function in the IPL by possibly interfering with pulmonary surfactant. We attempted to investigate this latter contention further by trying to observe the effect of administered cationic poly-peptide solutions in which the positive charge of the cations had been partially neutralised. For this phosphatidylglycerol was added to the poly-Arg solutions, but the viscosity of the mixtures (suspensions) obtained was such that it was difficult to administer them and these suspensions had a propensity to deposit in the upper branches of the airway (and not the alveoli) only. This line of investigation could therefore not be continued in the present study.

The effect that the cationic poly-peptides had on lung function may also be related to the ratios of proteins and cationic compounds to surfactant lipid attained in the alveoli. In their *in vitro* study, Kobayashi, et al (86) showed that nearly complete inhibition of

surfactant activity occurred at a protein to surfactant lipid ratio of 4.5, while under *in vivo* conditions the ratio was 11.2. The level of the cationic nature of the protein was however not established. In another study on the effects of cationic liposome-DNA complexes on pulmonary surfactant function, Pinar Boncuk et al (49) found that the lung-derived surfactants Curosurf$^{R}$ and Survanta$^{R}$ were susceptible to some inactivation *in vitro* when the cationic lipid /surfactant lipid ratio was greater than 1:5 (0.2). Based on the assumption of Jackson et al (87) that the lungs of adult rats contain 10 - 15 mg surfactant lipid per kg body weight, the rats (280 to 370 g body weight) used in this study had average surfactant lipid levels ranging between 3.5 to 4.6 mg surfactant lipid approximately. This means that the poly-peptide to surfactant lipid ratio obtained with the 0.0225, 0.225 and 2.25 mg doses of poly-Arg and poly-Lys were in the order of about 1:200, 1:20 and 1:2 (i.e. 0.005, 0.05 and 0.5), respectively. Even though these of cationic agent to anionic lipid ratios were much lower than that found in the *in vitro* study our data showed that as the poly-cations / surfactant lipid ratios increased the corresponding change in lung compliance (i.e. the most sensitive parameter in lung mechanics for assessing lung function) was non-significant ($P > 0.05$) for the 0.0225 mg dose group and significant ($P < 0.05$) for the 0.225 and 2.25 mg dose groups. Of course one cannot be sure that the ratios prevailed uniformly throughout the lung, but whether viewed in terms of poly-peptide to surfactant lipid ratio or simply in terms of dose of poly-peptide, we can conclude that the effects of the poly-peptides were distinctly dose-dependent.

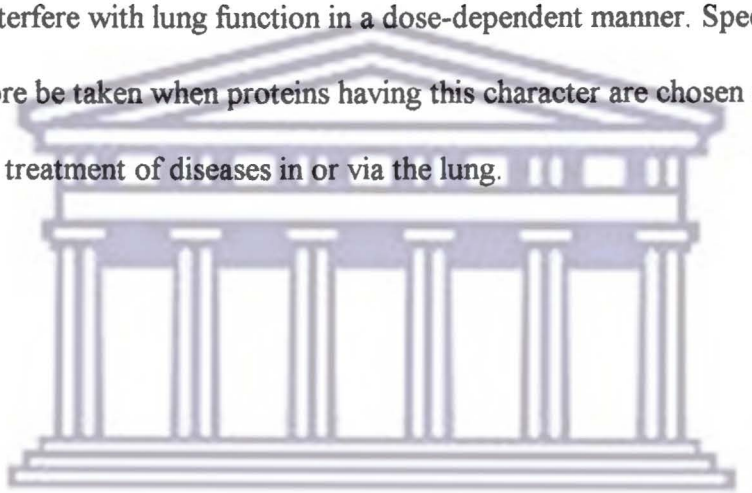
Finally, in addition to the effects on respiratory mechanics , the higher 0.225 and 2.25 mg doses of the cationic poly-peptides also induced severe edema in the lung. The

identification of the edema was based on the extent of increase of the lung weight and the visual observation of foamy liquid in the trachea. The increase in lung weight produced by these two doses of cationic poly-peptides appeared to be inversely proportional to the decrease in lung compliance and the increase in lung resistance. The greater the lung weight, the more severe the edema and the greater the decrease of lung compliance.

Unfortunately, it was not the intention with the present study to also explore the possible mechanism for the polymer-induced oedema formation. However, according to Greene (88) the hydrostatic pressure in the pulmonary capillary and tension of the surface film in the alveolus are opposed by the colloid osmotic pressure of the plasma proteins and the air pressure in the alveolus. These pressures are exerted across a thin tissue space sandwiched between the lumen of the capillary and the lumen of the alveolus. Changes in hydrostatic pressure, plasma oncotic pressure, capillary permeability and lymphatic drainage and the effect of surface-active agents may alter the balance of the above forces in the lung and thus result in pulmonary edema. Our results showed that, in the present study, the pulmonary arterial pressure remained stable during the occurrence of the edema (see appendix 6, and 7.2). Further, the perfusion medium (made according to the formula of Uhlig et al (89) ) and air and ventilation pressures used, as well as the lack of lymphatic drainage in the IPL, were not responsible for the edema as is evident from the lack of edema found in the saline-dosed lungs. Also it was unlikely that the high molecular weight poly-peptides migrated through the narrow pores of the alveolar epithelium (64,90) and tissue space to bind to the capillary wall and affect its permeability. A more likely explanation for

edema induced by the cationic poly-peptide may however involve an effect on alveolar surface tension. Indeed, it has been reported that pulmonary edema can be induced by increases in alveolar surface tension without significant change in colloid oncotic pressure and/or pulmonary microvascular hydrostatic pressure (91,92). In fact, such edema may be the consequence of reduced interstitial peri-microvascular hydrostatic pressure and consequent reduced resistance to fluid leakage from the capillaries, caused by the increased alveolar surface tension (93). An increase in surface tension also favours fluid leakage presumably because it increases the microvascular transmural pressure (94). An impairment in surfactant function which can lead to an increase in surface tension at the air-liquid interphase on the alveolar walls and an increased pressure gradient across the alveolar-capillary membrane, favouring further accumulation of protein-rich edema fluid into the alveolar space (95-100), may thus be considered as a viable mechanism for the poly-peptide induced edema found in the present study. Whether this is similar to the edema which occurs in ARDS when circulatory proteins leak into alveoli and impair surfactant function (34) needs to be studied further. Finally, the possibility exists that the poly-peptides can perhaps also have caused the edema as a results of a direct action on the epithelial cells. For example, Coyle et al (101) and Uchida et al (102) have shown that cationic proteins such as poly-lysine, human eosinophil-derived granule major basic protein (MBP), cathepsin G etc. may *in vivo* in the rat induce increase airway responsiveness as a result of a charge interaction with components of epithelial cells. The airway responsiveness were measured in terms of response to methacholine but they did not however monitor edema formation. Our results from the present study does not allow us to exclude this possible explanation for the occurrence of edema.

In present study the high molecular weight cationic poly-peptides at the relatively high doses administered into the lung induced significant changes of lung mechanics and lung edema. This is an important finding as far as the safety of pulmonary delivered drugs is concerned. As applies to any route of administration, great care must be exercised to ensure that inadvertent toxic effects do not result from the delivery of drug (or adjuvant) to the site of absorption (103). Our findings suggest that the pulmonary delivery of high molecular weight compounds (proteins) with positive charge may interfere with lung function in a dose-dependent manner. Special care should therefore be taken when proteins having this character are chosen as drugs (or adjuvants) for treatment of diseases in or via the lung.



# CHAPTER 7

## CONCLUSION

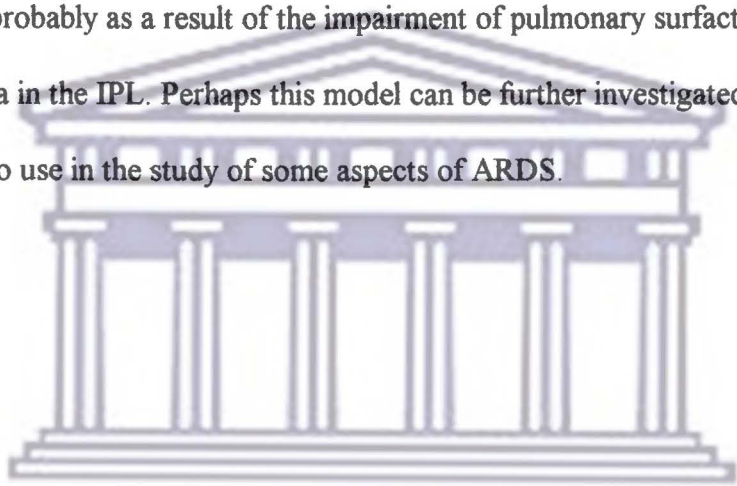
The objectives of this study were to (1) adapt the IPL so that it could be used to administer cationic polymers into the alveoli via the inhalation route and (2) to use the adapted model to investigate the effect which such inhaled cationic polymers could have on lung functions. The following conclusions may be drawn from the results of this investigation:

(1) With the use of intra-tracheal instillation and short duration positive pressure ventilation the IPL can be a suitable model to investigate the effects which inhaled poly-peptides might have on lung function. A more thorough comparative investigation of all three methods of administration considered, is however warranted, especially since this may help establish the IPL as an effective tool to investigate various aspects of the pulmonary pharmacodynamics and pharmacokinetics of inhaled pharmaceuticals.

(2) High molecular weight cationic poly-peptides, in contrast to anionic poly-peptides, administered into the alveoli of the IPL cause a significant dose-dependent deterioration in lung mechanics. It is speculated that these changes may arise as a result of an interaction between the cationic poly-peptides and the anionic components of pulmonary surfactant and a subsequent inactivation of the surfactant's surface tension lowering activity as was seen in previous *in*

*vitro* studies. We should thus be alert to the fact that the administration of protein drugs ( as pharmaceuticals), and especially those having a strong cationic nature, via the inhaled route, *in vivo* may also be expected to produce profound adverse effects on lung function. This should be the topic of urgent future investigation.

(3) The inhalation of high doses of high molecular weight cationic poly-peptides can, probably as a result of the impairment of pulmonary surfactant, lead to edema in the IPL. Perhaps this model can be further investigated as a potential tool to use in the study of some aspects of ARDS.



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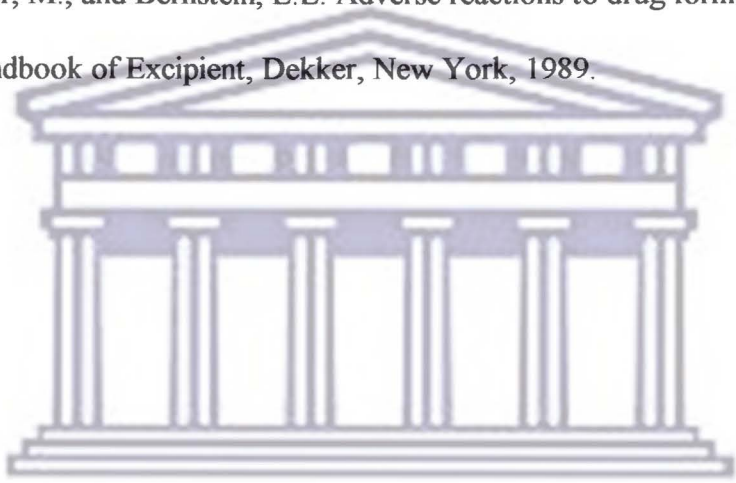
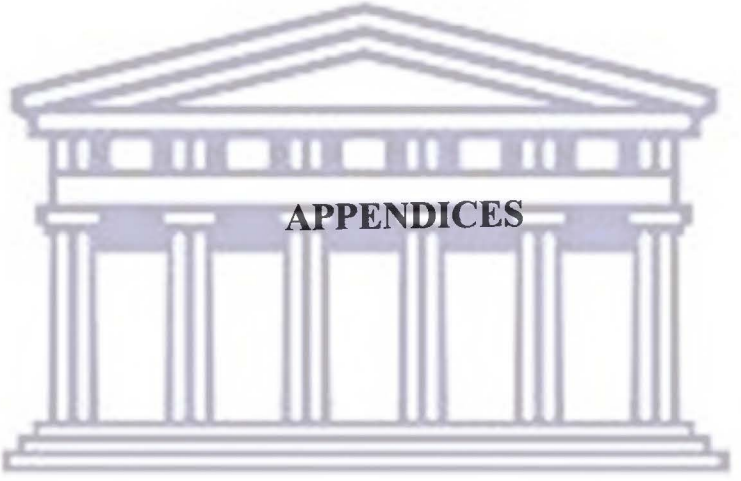


# APPENDICES



Appendix 1.1 The Effect of Different Polypeptides on Tidal Volume ($V_T$) in the IPL
( data for figure 13, page 60 )

| Polypeptides | $V_T$ during Equilibration Phase | | | $V_T$ during Post-Administration Phase | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 6 min | 10 min | 18 min | 22 min | 26 min | 36 min | 46 min |
| Saline-150µl | 1.44 ±0.035 | 1.402 ±0.026 | 1.382 ±.0.018 | 1.2 ±0.032 | 1.176 ±.0.049 | 1.134 ±0.04 | 1.066 ±0.043 | 0.998 ±0.077 |
| Poly-Glu-2.25mg/150µl | 1.38 ±0.02 | 1.352 ±0.022 | 1.344 ±0.023 | 1.254 ±0.047 | 1.248 ±0.044 | 1.194 ±0.055 | 1.14 ±0.04 | 1.06 ±0.05 |
| Poly-Lys-0.0225mg/150µl | 1.48 ± 0.02 | 1.416 ±0.031 | 1.388 ±0.022 | 1.2 ±0.032 | 1.142 ±0.028 | 1.12 ±0.037 | 1.01 ±0.04 | 0.966 ±0.051 |
| Poly-Lys-0.225mg/150µl | 1.4 ±0.032 | 1.336 ±0.048 | 1.312 ±0.052 | 0.96 ±0.108 | 0.88 ±0.116 | 0.84 ±0.126 | 0.6436* ±0.165 | 0.4046* ±0.12 |
| Poly-Lys-2.25mg/150µl | 1.36 ±0.04 | 1.036 ±0.048 | 1.3 ±0.045 | 0.79* ±0.071 | 0.68* ±0.077 | 0.472* ±0.113 | | |
| Poly-Arg-0.0225mg/150µl | 1.44 ±0.04 | 1.44 ±0.04 | 1.38 ±0.02 | 1.176 ±0.054 | 1.14 ±0.051 | 1.094 ±0.042 | 1.032 ±0.052 | 0.946 ±0.047 |
| Poly-Arg-0.225mg/150µl | 1.384 ±0.035 | 1.38 ±0.037 | 1.35 ±0.042 | 1.03 ±0.08 | 0.954 ±0.087 | 0.914 ±0.088 | 0.726* ±0.087 | 0.432* ±0.1 |
| Poly-Arg-2.25mg/150µl | 1.36 ±0.04 | 1.3 ±0.055 | 1.3 0.055 | 0.702* ±0.062 | 0.56* ±0.08 | 0.449* ±0.085 | | |

$V_T$ = tidal volume in ml ; each data point represents mean ± SE, n = 5; * Mann-Whitney U test , $P < 0.05$, alpha = 0.00714 for points during the 2min to 26min period and alpha = 0.01 for points at 36 and 46min.

Appendix 1.2 The Effect of Saline and Polypeptides on Tidal Volume ($V_T$) in the IPL

| Polypeptides | $V_T$ during Equilibration Phase | | | $V_T$ during Post-Administration Phase | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 6 min | 10 min | 18 min | 22 min | 26 min | 36 min | 46 min |
| Saline-150µl | 1.32 | 1.31 | 1.31 | 1.2 | 1.18 | 1.13 | 1.08 | 0.99 |
| | 1.4 | 1.4 | 1.4 | 1.2 | 1.2 | 1.16 | 1.1 | 1.1 |
| | 1.5 | 1.4 | 1.4 | 1.1 | 1 | 1 | 0.9 | 0.7 |
| | 1.48 | 1.45 | 1.4 | 1.2 | 1.2 | 1.13 | 1.1 | 1.1 |
| | 1.5 | 1.45 | 1.4 | 1.3 | 1.3 | 1.25 | 1.15 | 1.1 |
| Poly-Glu-2.25mg/150µl | 1.4 | 1.4 | 1.4 | 1.3 | 1.3 | 1.3 | 1.2 | 1.1 |
| | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.2 | 1.2 | 1.1 |
| | 1.4 | 1.36 | 1.32 | 1.1 | 1.1 | 1 | 1 | 0.9 |
| | 1.4 | 1.4 | 1.4 | 1.37 | 1.34 | 1.3 | 1.2 | 1.2 |
| | 1.4 | 1.3 | 1.3 | 1.2 | 1.2 | 1.17 | 1.1 | 1 |
| Poly-Lys-0.0225mg/150µl | 1.5 | 1.45 | 1.42 | 1.2 | 1.15 | 1.1 | 0.9 | 0.8 |
| | 1.5 | 1.46 | 1.4 | 1.1 | 1.06 | 1 | 0.95 | 0.96 |
| | 1.5 | 1.47 | 1.42 | 1.3 | 1.2 | 1.2 | 1.1 | 1.1 |
| | 1.4 | 1.3 | 1.3 | 1.2 | 1.1 | 1.1 | 1 | 0.93 |
| | 1.5 | 1.4 | 1.4 | 1.2 | 1.2 | 1.2 | 1.1 | 1.04 |
| Poly-Lys-0.225mg/150µl | 1.5 | 1.48 | 1.46 | 1.3 | 1.2 | 1.2 | 1.1 | 0.5 |
| | 1.4 | 1.3 | 1.2 | 0.7 | 0.6 | 0.55 | 0.4 | 0.2 |
| | 1.3 | 1.2 | 1.2 | 0.8 | 0.7 | 0.6 | 0.23 | 0.123 |
| | 1.4 | 1.3 | 1.3 | 0.9 | 0.8 | 0.8 | 0.538 | 0.4 |
| | 1.4 | 1.4 | 1.4 | 1.1 | 1.1 | 1.05 | 0.95 | 0.8 |
| Poly-Lys-2.25mg/150µl | 1.4 | 1.4 | 1.4 | 1 | 0.9 | 0.8 | | |
| | 1.5 | 1.43 | 1.4 | 0.9 | 0.8 | 0.66 | | |
| | 1.3 | 1.3 | 1.3 | 0.6 | 0.47 | 0.2 | | |
| | 1.3 | 1.2 | 1.2 | 0.8 | 0.65 | 0.277 | | |
| | 1.3 | 1.2 | 1.2 | 0.7 | 0.58 | 0.423 | | |
| Poly-Arg-0.0225mg/150µl | 1.3 | 1.3 | 1.3 | 1 | 1 | 1 | 1 | 0.9 |
| | 1.4 | 1.4 | 1.4 | 1.15 | 1.1 | 1.1 | 0.92 | 0.83 |
| | 1.5 | 1.5 | 1.4 | 1.3 | 1.3 | 1.2 | 1.2 | 1.1 |
| | 1.5 | 1.5 | 1.4 | 1.28 | 1.2 | 1.17 | 1.1 | 1 |
| | 1.5 | 1.5 | 1.4 | 1.15 | 1.1 | 1 | 0.94 | 0.9 |
| Poly-Arg-0.225mg/150µl | 1.4 | 1.4 | 1.3 | 0.9 | 0.8 | 0.74 | 0.53 | 0.26 |
| | 1.3 | 1.3 | 1.3 | 0.85 | 0.8 | 0.8 | 0.64 | 0.55 |
| | 1.32 | 1.3 | 1.27 | 1 | 0.9 | 0.83 | 0.6 | 0.4 |
| | 1.4 | 1.4 | 1.38 | 1.1 | 1 | 0.97 | 0.86 | 0.75 |
| | 1.5 | 1.5 | 1.5 | 1.3 | 1.27 | 1.23 | 1 | 0.2 |
| Poly-Arg-2.25mg/150µl | 1.4 | 1.3 | 1.3 | 0.8 | 0.7 | 0.69 | | |
| | 1.3 | 1.2 | 1.2 | 0.61 | 0.3 | 0.17 | | |
| | 1.3 | 1.3 | 1.3 | 0.6 | 0.55 | 0.52 | | |
| | 1.3 | 1.2 | 1.2 | 0.6 | 0.5 | 0.384 | | |
| | 1.5 | 1.5 | 1.5 | 0.9 | 0.75 | 0.48 | | |

$V_T$ = tidal volume in ml; spreadsheet of data collated from computer printouts document 1 and 2 (see methods section).

Appendix 2.1 The Effect of Different Polypeptides on Lung Resistance ($R_L$) in the IPL
( data for figure 14, page 62 )

| Polypeptides | $R_L$ during Equilibration Phase | | | $R_L$ during Post-Administration Phase | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 6 min | 10 min | 18 min | 22 min | 26 min | 36 min | 46 min |
| Saline-150µl | 0.354 ±0.004 | 0.3562 ±0.004 | 0.3578 ±0.005 | 0.368 ±0.005 | 0.3692 ±0.006 | 0.3728 ±0.005 | 0.384 ±0.009 | 0.3974 ±0.017 |
| Poly-Glu-2.25mg/150µl | 0.3518 ±0.004 | 0.353 ±0.005 | 0.3538 ±0.004 | 0.358 ±0.006 | 0.3588 ±0.006 | 0.3602 ±0.007 | 0.3682 ±0.008 | 0.375 ±0.009 |
| Poly-Lys-0.0225mg/150µl | 0.3654 ±0.008 | 0.3698 ±0.007 | 0.3734 ±0.008 | 0.385 ±0.007 | 0.3898 ±0.007 | 0.396 ±0.01 | 0.4086 ±0.012 | 0.4236 ±0.016 |
| Poly-Lys-0.225mg/150µl | 0.377 ±0.006 | 0.3808 ±0.007 | 0.3844 ±0.007 | 0.452 ±0.019 | 0.472 ±0.025 | 0.4812 ±0.026 | 0.5658* ±0.055 | 0.6808* ±0.058 |
| Poly-Lys-2.25mg/150µl | 0.3744 ±0.005 | 0.3756 ±0.006 | 0.3764 ±0.005 | 0.516* ±0.038 | 0.5844* ±0.064 | 0.6926* ±0.087 | | |
| Poly-Arg-0.0225mg/150µl | 0.3678 ±0.008 | 0.3682 ±0.008 | 0.3686 ±0.007 | 0.391 ±0.012 | 0.3922 ±0.012 | 0.3938 ±0.012 | 0.4034 ±0.033 | 0.4194 ±0.008 |
| Poly-Arg-0.225mg/150µl | 0.3876 ±0.004 | 0.3896 ±0.003 | 0.3934 ±0.004 | 0.443 ±0.012 | 0.458 ±0.015 | 0.4746 ±0.019 | 0.52* ±0.023 | 0.8092* ±0.107 |
| Poly-Arg-2.25mg/150µl | 0.3638 ±0.011 | 0.3688 ±0.011 | 0.3766 ±0.01 | 0.511* ±0.039 | 0.5562* ±0.054 | 0.672* ±0.081 | | |

$R_L$ = lung resistance in $cmH_2O.ml^{-1}.sec^{-1}$; each data point represents mean ± SE, n = 5; * Mann-Whitney U test, $P < 0.05$, alpha = 0.00714 for points during the 2min to 26min period and alpha = 0.01 for points at 36 and 46min.

Appendix 2.2 The Effect of Saline and Polypeptides on Lung Resistance ($R_L$) in the IPL

| Polypeptides | $R_L$ during Equilibration Phase | | | $R_L$ during Post-Administration Phase | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 6 min | 10 min | 18 min | 22 min | 26 min | 36 min | 46 min |
| Saline-150µl | 0.364 | 0.365 | 0.365 | 0.369 | 0.369 | 0.37 | 0.375 | 0.378 |
| | 0.348 | 0.348 | 0.348 | 0.35 | 0.351 | 0.36 | 0.367 | 0.369 |
| | 0.34 | 0.344 | 0.346 | 0.38 | 0.385 | 0.39 | 0.41 | 0.46 |
| | 0.36 | 0.365 | 0.37 | 0.378 | 0.379 | 0.38 | 0.4 | 0.41 |
| | 0.358 | 0.359 | 0.36 | 0.362 | 0.362 | 0.364 | 0.368 | 0.37 |
| Poly-Glu-2.25mg/150µl | 0.346 | 0.347 | 0.348 | 0.349 | 0.35 | 0.35 | 0.366 | 0.38 |
| | 0.341 | 0.342 | 0.343 | 0.344 | 0.346 | 0.347 | 0.349 | 0.352 |
| | 0.361 | 0.363 | 0.364 | 0.378 | 0.381 | 0.386 | 0.397 | 0.404 |
| | 0.354 | 0.355 | 0.356 | 0.356 | 0.356 | 0.356 | 0.357 | 0.358 |
| | 0.357 | 0.358 | 0.358 | 0.361 | 0.361 | 0.362 | 0.372 | 0.381 |
| Poly-Lys-0.0225mg/150µl | 0.382 | 0.385 | 0.389 | 0.399 | 0.403 | 0.407 | 0.44 | 0.471 |
| | 0.385 | 0.389 | 0.394 | 0.401 | 0.41 | 0.428 | 0.437 | 0.454 |
| | 0.35 | 0.353 | 0.356 | 0.365 | 0.372 | 0.378 | 0.386 | 0.396 |
| | 0.36 | 0.362 | 0.363 | 0.376 | 0.379 | 0.381 | 0.391 | 0.403 |
| | 0.35 | 0.36 | 0.365 | 0.384 | 0.385 | 0.386 | 0.389 | 0.394 |
| Poly-Lys-0.225mg/150µl | 0.37 | 0.372 | 0.375 | 0.388 | 0.401 | 0.407 | 0.414 | 0.7 |
| | 0.359 | 0.361 | 0.363 | 0.488 | 0.514 | 0.526 | 0.68 | 0.68 |
| | 0.38 | 0.382 | 0.383 | 0.46 | 0.475 | 0.487 | 0.611 | 0.654 |
| | 0.383 | 0.392 | 0.402 | 0.492 | 0.535 | 0.547 | 0.67 | 0.868 |
| | 0.393 | 0.397 | 0.399 | 0.43 | 0.435 | 0.439 | 0.454 | 0.502 |
| Poly-Lys-2.25mg/150µl | 0.359 | 0.36 | 0.361 | 0.41 | 0.44 | 0.47 | | |
| | 0.365 | 0.365 | 0.367 | 0.485 | 0.515 | 0.581 | | |
| | 0.386 | 0.388 | 0.389 | 0.61 | 0.8 | 0.951 | | |
| | 0.374 | 0.375 | 0.375 | 0.476 | 0.515 | 0.635 | | |
| | 0.386 | 0.39 | 0.39 | 0.599 | 0.652 | 0.826 | | |
| Poly-Arg-0.0225mg/150µl | 0.358 | 0.358 | 0.358 | 0.358 | 0.36 | 0.361 | 0.364 | 0.405 |
| | 0.361 | 0.362 | 0.362 | 0.386 | 0.387 | 0.389 | 0.415 | 0.424 |
| | 0.362 | 0.362 | 0.363 | 0.38 | 0.381 | 0.381 | 0.39 | 0.4 |
| | 0.398 | 0.399 | 0.399 | 0.429 | 0.43 | 0.43 | 0.432 | 0.411 |
| | 0.36 | 0.36 | 0.361 | 0.402 | 0.403 | 0.408 | 0.416 | 0.427 |
| Poly-Arg-0.225mg/150µl | 0.393 | 0.398 | 0.4 | 0.45 | 0.48 | 0.51 | 0.55 | 0.578 |
| | 0.39 | 0.392 | 0.394 | 0.48 | 0.5 | 0.52 | 0.557 | 0.562 |
| | 0.388 | 0.39 | 0.391 | 0.44 | 0.45 | 0.468 | 0.56 | 0.757 |
| | 0.385 | 0.39 | 0.404 | 0.44 | 0.45 | 0.46 | 0.486 | 0.519 |
| | 0.375 | 0.378 | 0.378 | 0.405 | 0.41 | 0.415 | 0.447 | 1.63 |
| Poly-Arg-2.25mg/150µl | 0.385 | 0.387 | 0.388 | 0.45 | 0.455 | 0.47 | | |
| | 0.365 | 0.37 | 0.37 | 0.49 | 0.506 | 0.68 | | |
| | 0.349 | 0.35 | 0.383 | 0.55 | 0.69 | 0.823 | | |
| | 0.39 | 0.397 | 0.402 | 0.64 | 0.68 | 0.877 | | |
| | 0.33 | 0.34 | 0.34 | 0.425 | 0.45 | 0.51 | | |

$R_L$ = Lung resistance in $cmH_2O.ml^{-1}.sec^{-1}$; spreadsheet of data collated from computer printouts document 1 and 2 (see methods section).

Appendix 3.1 The Effect of Different Polypeptides on Lung Compliance ($C_L$) in the IPL
( data for figure 15, page 63 )

| Polypeptides | $C_L$ during Equilibration Phase | | | $C_L$ during Post-Administration Phase | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 6 min | 10 min | 18 min | 22 min | 26 min | 36 min | 46 min |
| Saline-150µl | 0.518 ±0.012 | 0.496 ±0.017 | 0.476 ±0.019 | 0.43 ±0.033 | 0.401 ±0.039 | 0.374 ±0.037 | 0.312 ±0.032 | 0.272 ±0.035 |
| Poly-Glu-2.25mg/150µl | 0.502 ±0.021 | 0.478 ±0.02 | 0.464 ±0.017 | 0.402 ±0.031 | 0.358 ±0.028 | 0.346 ±0.031 | 0.283 ±0.021 | 0.251 ±0.02 |
| Poly-Lys-0.0225mg/150µl | 0.507 ±0.022 | 0.478 ±0.022 | 0.457 ±0.023 | 0.38 ±0.021 | 0.343 ±0.017 | 0.318 ±0.013 | 0.254 ±0.015 | 0.227 ±0.01 |
| Poly-Lys-0.225mg/150µl | 0.457 ±0.035 | 0.417 ±0.041 | 0.401 ±0.041 | 0.25* ±0.057 | 0.202* ±0.047 | 0.18* ±0.046 | 0.133* ±0.04 | 0.083* ±0.02 |
| Poly-Lys-2.25mg/150µl | 0.45 ±0.034 | 0.425 ±0.034 | 0.41 ±0.033 | 0.171* ±0.02 | 0.131* ±0.021 | 0.096* ±0.022 | | |
| Poly-Arg-0.0225mg/150µl | 0.517 ±0.025 | 0.495 ±0.025 | 0.475 ±0.021 | 0.345 ±0.028 | 0.319 ±0.024 | 0.312 ±0.03 | 0.258 ±0.047 | 0.221 ±0.025 |
| Poly-Arg-0.225mg/150µl | 0.47 ±0.035 | 0.454 ±0.04 | 0.445 ±0.041 | 0.297 ±0.041 | 0.26* ±0.034 | 0.216* ±0.029 | 0.158* ±0.023 | 0.086* ±0.018 |
| Poly-Arg-2.25mg/150µl | 0464 ±0.025 | 0.443 ±0.027 | 0.431 ±0.03 | 0.18* ±0.023 | 0.139* 0.022 | 0.102* ±0.019 | | |

$C_L$ = lung compliance in ml.cm$H_2O^{-1}$; each data point represents mean ± SE, n = 5; * Mann-Whitney U test , $P < 0.05$, alpha = 0.00714 for points during the 2min to 26min period and alpha = 0.01 for points at 36 and 46min.

Appendix 3.2 The Effect of Saline and Polypeptides on Lung Compliance ($C_L$) in the IPL

| Polypeptides | $C_L$ during Equilibration Phase | | | $C_L$ during Post-Administration Phase | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 6 min | 10 min | 18 min | 22 min | 26 min | 36 min | 46 min |
| Saline-150µl | 0.54 | 0.52 | 0.5 | 0.47 | 0.43 | 0.39 | 0.32 | 0.264 |
| | 0.52 | 0.51 | 0.5 | 0.46 | 0.45 | 0.43 | 0.36 | 0.35 |
| | 0.47 | 0.43 | 0.4 | 0.3 | 0.245 | 0.23 | 0.19 | 0.145 |
| | 0.53 | 0.52 | 0.5 | 0.47 | 0.44 | 0.42 | 0.36 | 0.32 |
| | 0.53 | 0.5 | 0.48 | 0.45 | 0.44 | 0.4 | 0.33 | 0.28 |
| Poly-Glu-2.25mg/150µl | 0.56 | 0.534 | 0.5 | 0.446 | 0.337 | 0.4 | 0.314 | 0.263 |
| | 0.47 | 0.458 | 0.439 | 0.431 | 0.395 | 0.362 | 0.315 | 0.289 |
| | 0.517 | 0.47 | 0.48 | 0.292 | 0.27 | 0.242 | 0.212 | 0.188 |
| | 0.53 | 0.512 | 0.49 | 0.461 | 0.436 | 0.412 | 0.317 | 0.293 |
| | 0.437 | 0.418 | 0.413 | 0.38 | 0.35 | 0.313 | 0.255 | 0.222 |
| Poly-Lys-0.0225mg/150µl | 0.525 | 0.47 | 0.425 | 0.417 | 0.385 | 0.351 | 0.29 | 0.25 |
| | 0.545 | 0.52 | 0.5 | 0.33 | 0.31 | 0.29 | 0.226 | 0.22 |
| | 0.55 | 0.528 | 0.517 | 0.431 | 0.37 | 0.34 | 0.278 | 0.24 |
| | 0.436 | 0.41 | 0.392 | 0.33 | 0.3 | 0.29 | 0.215 | 0.193 |
| | 0.477 | 0.46 | 0.449 | 0.39 | 0.35 | 0.32 | 0.26 | 0.23 |
| Poly-Lys-0.225mg/150µl | 0.581 | 0.572 | 0.561 | 0.46 | 0.36 | 0.33 | 0.256 | 0.099 |
| | 0.41 | 0.37 | 0.34 | 0.148 | 0.11 | 0.102 | 0.054 | 0.037 |
| | 0.4 | 0.365 | 0.35 | 0.163 | 0.125 | 0.108 | 0.066 | 0.056 |
| | 0.433 | 0.36 | 0.345 | 0.2 | 0.155 | 0.114 | 0.092 | 0.07 |
| | 0.46 | 0.42 | 0.411 | 0.28 | 0.26 | 0.245 | 0.198 | 0.152 |
| Poly-Lys-2.25mg/150µl | 0.528 | 0.521 | 0.499 | 0.23 | 0.19 | 0.167 | | |
| | 0.508 | 0.49 | 0.472 | 0.198 | 0.16 | 0.123 | | |
| | 0.4 | 0.351 | 0.338 | 0.11 | 0.069 | 0.039 | | |
| | 0.4 | 0.36 | 0.345 | 0.158 | 0.115 | 0.068 | | |
| | 0.42 | 0.405 | 0.395 | 0.161 | 0.12 | 0.082 | | |
| Poly-Arg-0.0225mg/150µl | 0.43 | 0.41 | 0.4 | 0.4 | 0.39 | 0.38 | 0.377 | 0.311 |
| | 0.54 | 0.52 | 0.51 | 0.4 | 0.338 | 0.3 | 0.223 | 0.197 |
| | 0.583 | 0.565 | 0.523 | 0.362 | 0.33 | 0.32 | 0.27 | 0.23 |
| | 0.51 | 0.492 | 0.473 | 0.302 | 0.29 | 0.28 | 0.238 | 0.202 |
| | 0.52 | 0.49 | 0.47 | 0.26 | 0.246 | 0.24 | 0.181 | 0.164 |
| Poly-Arg-0.225mg/150µl | 0.48 | 0.465 | 0.45 | 0.33 | 0.271 | 0.2 | 0.13 | 0.086 |
| | 0.47 | 0.46 | 0.451 | 0.275 | 0.26 | 0.2 | 0.17 | 0.128 |
| | 0.4 | 0.358 | 0.336 | 0.212 | 0.183 | 0.164 | 0.103 | 0.062 |
| | 0.42 | 0.41 | 0.401 | 0.23 | 0.205 | 0.187 | 0.149 | 0.121 |
| | 0.58 | 0.58 | 0.585 | 0.44 | 0.38 | 0.33 | 0.238 | 0.033 |
| Poly-Arg-2.25mg/150µl | 0.48 | 0.47 | 0.463 | 0.23 | 0.19 | 0.16 | | |
| | 0.48 | 0.45 | 0.44 | 0.15 | 0.1 | 0.057 | | |
| | 0.41 | 0.37 | 0.351 | 0.16 | 0.12 | 0.099 | | |
| | 0.42 | 0.4 | 0.382 | 0.122 | 0.09 | 0.064 | | |
| | 0.53 | 0.525 | 0.52 | 0.24 | 0.195 | 0.129 | | |

$C_L$ = Lung compliance in ml.cm$H_2O^{-1}$; spreadsheet of data collated from computer printouts document 1 and 2 (see methods section).

Appendix 4.1 Sample of Computer Printout of Document 1 Showing Tidal Volume (TV), Transpulmonary Pressure (PPLEU), Lung Resistance (RES) and Lung Compliance (COMPL) during Equilibration Period

Test HSE

| Time (Second) | MAXIN | MAXEX | TV | PPLEU | RES | COMPL |
|---|---|---|---|---|---|---|
| 10 | 6.1 | -5.8 | 1.7 | 10.1 | 0.355 | 0.467 |
| 20 | 6.1 | -5.8 | 1.7 | 10.1 | 0.352 | 0.469 |
| 30 | 6.1 | -5.8 | 1.7 | 10.1 | 0.356 | 0.467 |
| 40 | 6.1 | -5.8 | 1.7 | 10.1 | 0.356 | 0.465 |
| 50 | 6.1 | -5.8 | 1.7 | 10.1 | 0.357 | 0.475 |
| 60 | 6.1 | -5.8 | 1.7 | 10.1 | 0.354 | 0.477 |
| 70 | 6.1 | -5.8 | 1.7 | 10.1 | 0.355 | 0.469 |
| 80 | 6.1 | -5.8 | 1.7 | 10.0 | 0.357 | 0.472 |
| 90 | 6.1 | -5.8 | 1.7 | 10.1 | 0.354 | 0.467 |
| 100 | 6.1 | -5.8 | 1.7 | 10.1 | 0.355 | 0.474 |
| 110 | 6.1 | -5.8 | 1.7 | 10.1 | 0.352 | 0.481 |
| 120 | 6.1 | -5.8 | 1.7 | 10.1 | 0.356 | 0.480 |
| 130 | 6.1 | -5.8 | 1.7 | 10.1 | 0.352 | 0.467 |
| 140 | 6.1 | -5.8 | 1.7 | 10.1 | 0.354 | 0.470 |
| 150 | 6.1 | -5.8 | 1.7 | 10.0 | 0.351 | 0.475 |
| 160 | 6.1 | -5.8 | 1.7 | 10.1 | 0.354 | 0.467 |
| 170 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.481 |
| 180 | 6.1 | -5.8 | 1.7 | 10.1 | 0.352 | 0.458 |
| 190 | 6.1 | -5.8 | 1.7 | 10.1 | 0.351 | 0.468 |
| 200 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.480 |
| 210 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.477 |
| 220 | 6.1 | -5.8 | 1.7 | 10.1 | 0.351 | 0.475 |
| 230 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.450 |
| 240 | 6.1 | -5.8 | 1.7 | 10.1 | 0.351 | 0.466 |
| 250 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.456 |
| 260 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.468 |
| 270 | 6.1 | -5.8 | 1.7 | 10.1 | 0.351 | 0.463 |
| 280 | 6.1 | -5.8 | 1.7 | 10.1 | 0.351 | 0.456 |
| 290 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.471 |
| 300 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.452 |
| 310 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.461 |
| 320 | 6.1 | -5.8 | 1.7 | 10.0 | 0.351 | 0.464 |
| 330 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.451 |
| 340 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.460 |
| 350 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.470 |
| 360 | 6.1 | -5.8 | 1.7 | 10.1 | 0.351 | 0.446 |
| 370 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.457 |
| 380 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.470 |
| 390 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.452 |
| 400 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.462 |
| 410 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.449 |
| 420 | 6.1 | -5.8 | 1.7 | 10.1 | 0.351 | 0.460 |
| 430 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.476 |
| 440 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.451 |
| 450 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.464 |
| 460 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.467 |
| 470 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.449 |
| 480 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.456 |
| 490 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.452 |
| 500 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.452 |
| 510 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.472 |
| 520 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.450 |
| 530 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.441 |
| 540 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.456 |
| 550 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.446 |
| 560 | 6.1 | -5.8 | 1.7 | 10.0 | 0.350 | 0.457 |
| 570 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.446 |
| 580 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.459 |
| 590 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.446 |
| 600 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.442 |
| 610 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.446 |
| 620 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.455 |
| 630 | 6.1 | -5.8 | 1.7 | 10.1 | 0.350 | 0.456 |
| 640 | 6.1 | -5.8 | 1.7 | 10.1 | 0.349 | 0.454 |
| 650 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.456 |
| 660 | 6.1 | -5.8 | 1.7 | 10.1 | 0.348 | 0.455 |

Appendix 4.2 Sample of Computer Printout of Document 2 Showing Tidal Volume (TV), Transpulmonary Pressure (PPLEU), Lung Resistance (RES) and Lung Compliance (COMPL) after the Administration of Saline

Test HSE

| Time (Second) | MAXIN | MAXEX | TV | PPLEU | RES | COMPL |
|---|---|---|---|---|---|---|
| 10 | 6.1 | -6.0 | 1.3 | 10.1 | 0.339 | 0.438 |
| 20 | 6.1 | -6.1 | 1.3 | 10.1 | 0.339 | 0.448 |
| 30 | 6.1 | -6.1 | 1.3 | 10.1 | 0.339 | 0.452 |
| 40 | 6.1 | -6.1 | 1.3 | 10.1 | 0.339 | 0.433 |
| 50 | 6.1 | -6.1 | 1.3 | 10.1 | 0.340 | 0.437 |
| 60 | 6.1 | -6.0 | 1.3 | 10.1 | 0.339 | 0.435 |
| 70 | 6.1 | -6.1 | 1.3 | 10.1 | 0.340 | 0.449 |
| 80 | 6.1 | -6.1 | 1.3 | 10.1 | 0.340 | 0.448 |
| 90 | 6.1 | -6.1 | 1.3 | 10.1 | 0.340 | 0.438 |
| 100 | 6.1 | -6.1 | 1.3 | 10.1 | 0.339 | 0.435 |
| 110 | 6.1 | -6.1 | 1.3 | 10.1 | 0.340 | 0.436 |
| 120 | 6.1 | -6.1 | 1.3 | 10.1 | 0.339 | 0.436 |
| 130 | 6.1 | -6.1 | 1.3 | 10.1 | 0.340 | 0.421 |
| 140 | 6.1 | -6.1 | 1.3 | 10.1 | 0.339 | 0.425 |
| 150 | 6.1 | -6.1 | 1.3 | 10.1 | 0.340 | 0.433 |
| 160 | 6.1 | -6.1 | 1.3 | 10.1 | 0.342 | 0.425 |
| 170 | 6.1 | -6.1 | 1.3 | 10.1 | 0.341 | 0.404 |
| 180 | 6.0 | -6.1 | 1.3 | 10.1 | 0.340 | 0.347 |
| 190 | 6.1 | -6.0 | 1.3 | 10.1 | 0.341 | 0.427 |
| 200 | 6.1 | -6.1 | 1.3 | 10.1 | 0.338 | 0.414 |
| 210 | 6.1 | -6.0 | 1.3 | 10.1 | 0.341 | 0.413 |
| 220 | 6.1 | -6.1 | 1.3 | 10.1 | 0.341 | 0.420 |
| 230 | 6.1 | -6.0 | 1.3 | 10.1 | 0.342 | 0.417 |
| 240 | 6.1 | -6.0 | 1.3 | 10.1 | 0.342 | 0.417 |
| 250 | 6.1 | -6.1 | 1.3 | 10.1 | 0.340 | 0.427 |
| 260 | 6.1 | -6.0 | 1.3 | 10.1 | 0.341 | 0.398 |
| 270 | 6.1 | -6.1 | 1.3 | 10.2 | 0.341 | 0.406 |
| 280 | 6.0 | -6.1 | 1.3 | 10.1 | 0.341 | 0.405 |
| 290 | 6.0 | -6.0 | 1.3 | 10.1 | 0.342 | 0.391 |
| 300 | 6.1 | -6.0 | 1.3 | 10.2 | 0.343 | 0.347 |
| 310 | 6.1 | -6.0 | 1.3 | 10.1 | 0.340 | 0.416 |
| 320 | 6.1 | -6.1 | 1.3 | 10.2 | 0.340 | 0.400 |
| 330 | 6.0 | -6.0 | 1.3 | 10.1 | 0.342 | 0.389 |
| 340 | 6.1 | -6.0 | 1.3 | 10.1 | 0.340 | 0.409 |
| 350 | 6.1 | -6.0 | 1.3 | 10.1 | 0.341 | 0.397 |
| 360 | 6.1 | -6.0 | 1.3 | 10.1 | 0.342 | 0.395 |
| 370 | 6.1 | -6.0 | 1.3 | 10.1 | 0.342 | 0.405 |
| 380 | 6.1 | -6.0 | 1.3 | 10.1 | 0.342 | 0.379 |
| 390 | 6.0 | -6.0 | 1.3 | 10.1 | 0.342 | 0.393 |
| 400 | 6.0 | -6.0 | 1.3 | 10.2 | 0.342 | 0.381 |
| 410 | 6.0 | -6.0 | 1.3 | 10.1 | 0.341 | 0.380 |
| 420 | 6.0 | -6.0 | 1.2 | 10.1 | 0.343 | 0.388 |
| 430 | 6.1 | -6.0 | 1.3 | 10.1 | 0.342 | 0.387 |
| 440 | 6.0 | -6.0 | 1.3 | 10.1 | 0.343 | 0.386 |
| 450 | 6.0 | -6.0 | 1.3 | 10.1 | 0.342 | 0.389 |
| 460 | 6.0 | -6.0 | 1.3 | 10.2 | 0.341 | 0.384 |
| 470 | 6.0 | -6.0 | 1.2 | 10.1 | 0.343 | 0.387 |
| 480 | 6.0 | -6.0 | 1.3 | 10.1 | 0.342 | 0.376 |
| 490 | 6.0 | -6.0 | 1.2 | 10.2 | 0.342 | 0.387 |
| 500 | 6.1 | -6.0 | 1.3 | 10.2 | 0.343 | 0.376 |
| 510 | 6.0 | -6.0 | 1.2 | 10.2 | 0.343 | 0.379 |
| 520 | 6.0 | -6.0 | 1.2 | 10.2 | 0.342 | 0.369 |
| 530 | 6.0 | -6.0 | 1.2 | 10.2 | 0.343 | 0.371 |
| 540 | 6.0 | -6.0 | 1.2 | 10.2 | 0.342 | 0.374 |

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## Appendix 4.2 (continue)

| Time (Second) | MAXIN | MAXEX | TV | APLEW | RSE | COMPL |
|---|---|---|---|---|---|---|
| 550 | 6.0 | -6.0 | 1.2 | 10.1 | 0.342 | 0.370 |
| 560 | 6.0 | -6.0 | 1.2 | 10.2 | 0.341 | 0.400 |
| 570 | 6.0 | -5.9 | 1.2 | 10.1 | 0.343 | 0.364 |
| 580 | 6.0 | -6.0 | 1.2 | 10.1 | 0.343 | 0.374 |
| 590 | 6.0 | -6.0 | 1.2 | 10.2 | 0.343 | 0.36- |
| 600 | 6.0 | -6.0 | 1.2 | 10.1 | 0.347 | 0.377 |
| 610 | 6.0 | -6.0 | 1.2 | 10.1 | 0.343 | 0.368 |
| 620 | 6.0 | -6.0 | 1.2 | 10.2 | 0.344 | 0.366 |
| 630 | 6.0 | -5.9 | 1.2 | 10.1 | 0.343 | 0.364 |
| 640 | 6.0 | -6.0 | 1.2 | 10.2 | 0.344 | 0.362 |
| 650 | 6.0 | -6.0 | 1.2 | 10.2 | 0.344 | 0.365 |
| 660 | 6.0 | -6.0 | 1.2 | 10.2 | 0.343 | 0.314 |
| 670 | 6.0 | -5.9 | 1.2 | 10.2 | 0.343 | 0.373 |
| 680 | 6.0 | -6.0 | 1.2 | 10.2 | 0.343 | 0.357 |
| 690 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.378 |
| 700 | 6.0 | -5.9 | 1.2 | 10.2 | 0.343 | 0.359 |
| 710 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.351 |
| 720 | 6.0 | -6.0 | 1.2 | 10.2 | 0.344 | 0.371 |
| 730 | 6.0 | -5.9 | 1.2 | 10.2 | 0.343 | 0.344 |
| 740 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.359 |
| 750 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.355 |
| 760 | 6.0 | -5.9 | 1.2 | 10.2 | 0.343 | 0.337 |
| 770 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.359 |
| 780 | 6.0 | -5.9 | 1.2 | 10.2 | 0.343 | 0.309 |
| 790 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.368 |
| 800 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.358 |
| 810 | 6.0 | -5.9 | 1.2 | 10.2 | 0.345 | 0.346 |
| 820 | 6.0 | -5.9 | 1.2 | 10.2 | 0.343 | 0.366 |
| 830 | 6.0 | -5.9 | 1.2 | 10.2 | 0.346 | 0.340 |
| 840 | 6.0 | -5.9 | 1.2 | 10.2 | 0.345 | 0.354 |
| 850 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.350 |
| 860 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.332 |
| 870 | 6.0 | -5.9 | 1.2 | 10.2 | 0.344 | 0.356 |
| 880 | 5.9 | -5.9 | 1.2 | 10.2 | 0.345 | 0.342 |
| 890 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.351 |
| 900 | 6.0 | -5.9 | 1.2 | 10.2 | 0.345 | 0.308 |
| 910 | 6.0 | -6.0 | 1.2 | 10.2 | 0.345 | 0.346 |
| 920 | 6.0 | -5.9 | 1.2 | 10.2 | 0.347 | 0.359 |
| 930 | 6.0 | -5.9 | 1.2 | 10.2 | 0.346 | 0.334 |
| 940 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.338 |
| 950 | 6.0 | -5.9 | 1.2 | 10.2 | 0.345 | 0.342 |
| 960 | 5.9 | -5.9 | 1.2 | 10.2 | 0.344 | 0.327 |
| 970 | 5.9 | -5.9 | 1.2 | 10.2 | 0.345 | 0.348 |
| 980 | 5.9 | -5.9 | 1.2 | 10.2 | 0.345 | 0.332 |
| 990 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.333 |
| 1000 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.337 |
| 1010 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.323 |
| 1020 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.297 |
| 1030 | 6.0 | -5.9 | 1.2 | 10.2 | 0.346 | 0.330 |
| 1040 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.319 |
| 1050 | 6.0 | -5.8 | 1.2 | 10.2 | 0.346 | 0.342 |
| 1060 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.329 |
| 1070 | 5.9 | -5.8 | 1.2 | 10.2 | 0.347 | 0.327 |
| 1080 | 5.9 | -5.8 | 1.2 | 10.2 | 0.347 | 0.338 |

Appendix 4.2 (continue)

| Time (Second) | MAXIN | MAXEX | TV | PPLEU | PEE | COMPL |
|---|---|---|---|---|---|---|
| 1090 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.327 |
| 1100 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.321 |
| 1110 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.319 |
| 1120 | 5.9 | -5.9 | 1.2 | 10.3 | 0.347 | 0.321 |
| 1130 | 5.9 | -5.8 | 1.2 | 10.2 | 0.347 | 0.318 |
| 1140 | 5.9 | -5.9 | 1.2 | 10.2 | 0.348 | 0.275 |
| 1150 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.347 |
| 1160 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.321 |
| 1170 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.317 |
| 1180 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.322 |
| 1190 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.322 |
| 1200 | 5.9 | -5.9 | 1.2 | 10.2 | 0.348 | 0.329 |
| 1210 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.306 |
| 1220 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.316 |
| 1230 | 5.9 | -5.9 | 1.2 | 10.2 | 0.347 | 0.322 |
| 1240 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.313 |
| 1250 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.304 |
| 1260 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.295 |
| 1270 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.321 |
| 1280 | 5.9 | -5.9 | 1.2 | 10.3 | 0.348 | 0.309 |
| 1290 | 5.9 | -5.8 | 1.2 | 10.2 | 0.347 | 0.323 |
| 1300 | 5.9 | -5.8 | 1.2 | 10.2 | 0.347 | 0.326 |
| 1310 | 5.9 | -5.8 | 1.2 | 10.2 | 0.347 | 0.315 |
| 1320 | 5.9 | -5.8 | 1.2 | 10.2 | 0.347 | 0.305 |
| 1330 | 5.9 | -5.8 | 1.2 | 10.3 | 0.347 | 0.307 |
| 1340 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.320 |
| 1350 | 5.9 | -5.8 | 1.2 | 10.3 | 0.347 | 0.317 |
| 1360 | 5.8 | -5.9 | 1.2 | 10.2 | 0.347 | 0.305 |
| 1370 | 5.9 | -5.8 | 1.2 | 10.3 | 0.347 | 0.308 |
| 1380 | 5.9 | -5.8 | 1.2 | 10.3 | 0.347 | 0.297 |
| 1390 | 5.9 | -5.9 | 1.2 | 10.2 | 0.348 | 0.311 |
| 1400 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.304 |
| 1410 | 5.9 | -5.9 | 1.2 | 10.2 | 0.348 | 0.302 |
| 1420 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.319 |
| 1430 | 5.9 | -5.8 | 1.2 | 10.3 | 0.348 | 0.313 |
| 1440 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.307 |
| 1450 | 5.9 | -5.9 | 1.2 | 10.2 | 0.346 | 0.296 |
| 1460 | 5.8 | -5.8 | 1.2 | 10.2 | 0.347 | 0.302 |
| 1470 | 5.8 | -5.8 | 1.1 | 10.2 | 0.348 | 0.309 |
| 1480 | 5.9 | -5.8 | 1.1 | 10.2 | 0.348 | 0.312 |
| 1490 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.308 |
| 1500 | 5.9 | -5.8 | 1.1 | 10.2 | 0.349 | 0.270 |
| 1510 | 5.9 | -5.8 | 1.2 | 10.2 | 0.348 | 0.307 |
| 1520 | 5.9 | -5.8 | 1.2 | 10.2 | 0.349 | 0.294 |
| 1530 | 5.9 | -5.8 | 1.1 | 10.2 | 0.349 | 0.295 |
| 1540 | 5.9 | -5.8 | 1.1 | 10.3 | 0.348 | 0.297 |
| 1550 | 5.9 | -5.8 | 1.1 | 10.3 | 0.350 | 0.299 |
| 1560 | 5.9 | -5.8 | 1.1 | 10.3 | 0.349 | 0.298 |
| 1570 | 5.8 | -5.8 | 1.1 | 10.2 | 0.348 | 0.310 |
| 1580 | 5.8 | -5.8 | 1.1 | 10.3 | 0.349 | 0.301 |
| 1590 | 5.8 | -5.8 | 1.1 | 10.3 | 0.348 | 0.302 |
| 1600 | 5.8 | -5.8 | 1.1 | 10.2 | 0.349 | 0.300 |
| 1610 | 5.8 | -5.8 | 1.1 | 10.2 | 0.349 | 0.300 |
| 1620 | 5.8 | -5.8 | 1.1 | 10.2 | 0.349 | 0.276 |

Appendix 4.2 (continued)

| Time | (Second) | MAXIN | MAXEX | TV | PPLEU | FES | COMPL |
|---|---|---|---|---|---|---|---|
| 1630 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.348 | 0.304 |
| 1640 | | 5.8 | -5.8 | 1.1 | 10.3 | 0.345 | 0.301 |
| 1650 | | 5.8 | -5.8 | 1.1 | 10.3 | 0.349 | 0.299 |
| 1660 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.350 | 0.297 |
| 1670 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.350 | 0.292 |
| 1680 | | 5.8 | -5.8 | 1.1 | 10.3 | 0.349 | 0.287 |
| 1690 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.351 | 0.286 |
| 1700 | | 5.8 | -5.8 | 1.1 | 10.3 | 0.349 | 0.286 |
| 1710 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.350 | 0.282 |
| 1720 | | 5.8 | -5.7 | 1.1 | 10.2 | 0.350 | 0.288 |
| 1730 | | 5.8 | -5.8 | 1.1 | 10.2 | 0.348 | 0.289 |
| 1740 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.349 | 0.286 |
| 1750 | | 5.8 | -5.8 | 1.1 | 10.2 | 0.352 | 0.307 |
| 1760 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.351 | 0.291 |
| 1770 | | 5.8 | -5.7 | 1.1 | 10.2 | 0.351 | 0.302 |
| 1780 | | 5.9 | -5.7 | 1.1 | 10.3 | 0.352 | 0.296 |
| 1790 | | 5.8 | -5.8 | 1.1 | 10.2 | 0.351 | 0.297 |
| 1800 | | 5.9 | -5.7 | 1.1 | 10.3 | 0.350 | 0.296 |
| 1810 | | 5.8 | -5.7 | 1.1 | 10.3 | 0.352 | 0.294 |
| 1820 | | 5.8 | -5.8 | 1.1 | 10.3 | 0.350 | 0.297 |
| 1830 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.352 | 0.292 |
| 1840 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.350 | 0.297 |
| 1850 | | 5.8 | -5.7 | 1.1 | 10.3 | 0.347 | 0.287 |
| 1860 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.354 | 0.263 |
| 1870 | | 5.8 | -5.7 | 1.1 | 10.3 | 0.350 | 0.297 |
| 1880 | | 5.9 | -5.8 | 1.1 | 10.3 | 0.351 | 0.293 |
| 1890 | | 5.9 | -5.7 | 1.1 | 10.3 | 0.350 | 0.264 |

Appendix 4.3 Sample of Computer Printout of Document 2 Showing Tidal Volume (TV), Transpulmonary Pressure (PPLEU), Lung Resistance (RES) and Lung Compliance (COMPL) after the Administration of 0.225 mg Poly-Lysine

| Time | (Second) | MAXIN | MAXEX | TV | PPLEU | RES | COMPL |
|---|---|---|---|---|---|---|---|
| 10 | | 5.3 | -4.5 | 0.9 | 10.5 | 0.492 | 0.200 |
| 20 | | 5.2 | -4.5 | 0.8 | 10.5 | 0.495 | 0.198 |
| 30 | | 5.1 | -4.5 | 0.8 | 10.5 | 0.497 | 0.202 |
| 40 | | 5.1 | -4.4 | 0.8 | 10.5 | 0.502 | 0.194 |
| 50 | | 5.1 | -4.4 | 0.9 | 10.5 | 0.508 | 0.189 |
| 60 | | 5.1 | -4.4 | 0.9 | 10.5 | 0.507 | 0.219 |
| 70 | | 5.1 | -4.4 | 0.9 | 10.5 | 0.515 | 0.178 |
| 80 | | 5.0 | -4.3 | 0.8 | 10.6 | 0.525 | 0.179 |
| 90 | | 5.0 | -4.3 | 0.9 | 10.5 | 0.520 | 0.176 |
| 100 | | 5.0 | -4.3 | 0.9 | 10.5 | 0.523 | 0.164 |
| 110 | | 5.0 | -4.3 | 0.9 | 10.5 | 0.523 | 0.179 |
| 120 | | 5.0 | -4.3 | 0.9 | 10.5 | 0.518 | 0.176 |
| 130 | | 5.0 | -4.3 | 0.9 | 10.5 | 0.524 | 0.177 |
| 140 | | 4.9 | -4.3 | 0.9 | 10.5 | 0.522 | 0.175 |
| 150 | | 4.9 | -4.2 | 0.9 | 10.5 | 0.531 | 0.174 |
| 160 | | 4.9 | -4.3 | 0.9 | 10.5 | 0.526 | 0.171 |
| 170 | | 4.9 | -4.3 | 0.8 | 10.6 | 0.533 | 0.171 |
| 180 | | 4.9 | -4.2 | 0.9 | 10.5 | 0.529 | 0.199 |
| 190 | | 4.8 | -4.2 | 0.8 | 10.5 | 0.533 | 0.170 |
| 200 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.544 | 0.169 |
| 210 | | 4.8 | -4.2 | 0.8 | 10.6 | 0.536 | 0.163 |
| 220 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.532 | 0.152 |
| 230 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.535 | 0.165 |
| 240 | | 4.9 | -4.2 | 0.8 | 10.5 | 0.535 | 0.191 |
| 250 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.533 | 0.161 |
| 260 | | 4.9 | -4.2 | 0.8 | 10.5 | 0.534 | 0.163 |
| 270 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.539 | 0.161 |
| 280 | | 4.9 | -4.1 | 0.8 | 10.5 | 0.534 | 0.159 |
| 290 | | 4.8 | -4.2 | 0.8 | 10.6 | 0.541 | 0.160 |
| 300 | | 4.8 | -4.2 | 0.8 | 10.5 | 0.541 | 0.189 |
| 310 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.532 | 0.156 |
| 320 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.544 | 0.158 |
| 330 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.540 | 0.158 |
| 340 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.543 | 0.144 |
| 350 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.527 | 0.159 |
| 360 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.534 | 0.186 |
| 370 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.534 | 0.157 |
| 380 | | 4.8 | -4.2 | 0.8 | 10.6 | 0.537 | 0.155 |
| 390 | | 4.9 | -4.2 | 0.8 | 10.5 | 0.534 | 0.152 |
| 400 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.533 | 0.156 |
| 410 | | 4.9 | -4.1 | 0.8 | 10.6 | 0.538 | 0.150 |
| 420 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.535 | 0.180 |
| 430 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.533 | 0.157 |
| 440 | | 4.9 | -4.1 | 0.8 | 10.6 | 0.541 | 0.148 |
| 450 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.538 | 0.153 |
| 460 | | 4.9 | -4.2 | 0.8 | 10.6 | 0.540 | 0.141 |
| 470 | | 4.9 | -4.1 | 0.8 | 10.6 | 0.537 | 0.153 |
| 480 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.537 | 0.176 |
| 490 | | 4.9 | -4.1 | 0.8 | 10.6 | 0.538 | 0.151 |
| 500 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.543 | 0.147 |
| 510 | | 4.7 | -4.1 | 0.8 | 10.6 | 0.541 | 0.147 |
| 520 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.545 | 0.147 |
| 530 | | 4.7 | -4.1 | 0.8 | 10.6 | 0.549 | 0.144 |
| 540 | | 4.8 | -4.1 | 0.8 | 10.6 | 0.546 | 0.148 |

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Appendix 4.3 (continue)

| (Second) | MAXIN | MAXSE | TO | PRED | SEE | COMPL |
|---|---|---|---|---|---|---|
| 550 | 4.3 | -4.1 | 0.8 | 10.6 | 0.547 | 0.140 |
| 560 | 4.7 | -4.1 | 0.8 | 10.6 | 0.550 | 0.140 |
| 570 | 4.7 | -4.0 | 0.8 | 10.6 | 0.547 | 0.140 |
| 580 | 4.7 | -4.0 | 0.8 | 10.6 | 0.547 | 0.138 |
| 590 | 4.7 | -4.1 | 0.8 | 10.6 | 0.554 | 0.140 |
| 600 | 4.7 | -4.1 | 0.8 | 10.6 | 0.547 | 0.139 |
| 610 | 4.7 | -4.1 | 0.8 | 10.6 | 0.550 | 0.137 |
| 620 | 4.7 | -4.1 | 0.8 | 10.6 | 0.550 | 0.141 |
| 630 | 4.7 | -4.0 | 0.8 | 10.6 | 0.543 | 0.135 |
| 640 | 4.7 | -4.1 | 0.7 | 10.6 | 0.551 | 0.136 |
| 650 | 4.7 | -4.0 | 0.8 | 10.6 | 0.550 | 0.136 |
| 660 | 4.7 | -4.1 | 0.7 | 10.6 | 0.550 | 0.141 |
| 670 | 4.7 | -4.1 | 0.7 | 10.6 | 0.550 | 0.131 |
| 680 | 4.7 | -4.0 | 0.7 | 10.6 | 0.555 | 0.134 |
| 690 | 4.7 | -4.0 | 0.7 | 10.6 | 0.558 | 0.130 |
| 700 | 4.6 | -4.0 | 0.7 | 10.6 | 0.559 | 0.128 |
| 710 | 4.7 | -4.0 | 0.8 | 10.6 | 0.561 | 0.130 |
| 720 | 4.7 | -4.0 | 0.7 | 10.6 | 0.556 | 0.139 |
| 730 | 4.6 | -4.0 | 0.7 | 10.6 | 0.562 | 0.126 |
| 740 | 4.6 | -4.0 | 0.7 | 10.6 | 0.561 | 0.128 |
| 750 | 4.6 | -4.0 | 0.7 | 10.7 | 0.562 | 0.126 |
| 760 | 4.6 | -4.0 | 0.7 | 10.6 | 0.563 | 0.125 |
| 770 | 4.6 | -4.0 | 0.7 | 10.6 | 0.557 | 0.128 |
| 780 | 4.6 | -4.0 | 0.7 | 10.6 | 0.556 | 0.144 |
| 790 | 4.6 | -4.0 | 0.7 | 10.6 | 0.561 | 0.127 |
| 800 | 4.6 | -4.0 | 0.7 | 10.6 | 0.560 | 0.121 |
| 810 | 4.6 | -4.0 | 0.7 | 10.6 | 0.560 | 0.127 |
| 820 | 4.6 | -4.0 | 0.7 | 10.6 | 0.559 | 0.112 |
| 830 | 4.6 | -4.0 | 0.7 | 10.6 | 0.563 | 0.119 |
| 840 | 4.6 | -3.9 | 0.7 | 10.6 | 0.564 | 0.131 |
| 850 | 4.5 | -3.9 | 0.7 | 10.7 | 0.573 | 0.112 |
| 860 | 4.5 | -3.9 | 0.7 | 10.6 | 0.574 | 0.115 |
| 870 | 4.5 | -3.9 | 0.7 | 10.7 | 0.571 | 0.111 |
| 880 | 4.5 | -3.9 | 0.7 | 10.6 | 0.578 | 0.112 |
| 890 | 4.5 | -3.8 | 0.7 | 10.7 | 0.574 | 0.108 |
| 900 | 4.4 | -3.9 | 0.7 | 10.7 | 0.578 | 0.127 |
| 910 | 4.5 | -3.8 | 0.6 | 10.6 | 0.585 | 0.108 |
| 920 | 4.4 | -3.8 | 0.7 | 10.6 | 0.584 | 0.108 |
| 930 | 4.4 | -3.8 | 0.7 | 10.6 | 0.587 | 0.108 |
| 940 | 4.4 | -3.8 | 0.7 | 10.6 | 0.587 | 0.106 |
| 950 | 4.4 | -3.8 | 0.7 | 10.6 | 0.584 | 0.109 |
| 960 | 4.4 | -3.8 | 0.7 | 10.7 | 0.588 | 0.103 |
| 970 | 4.4 | -3.8 | 0.7 | 10.6 | 0.589 | 0.107 |
| 980 | 4.3 | -3.8 | 0.6 | 10.6 | 0.596 | 0.105 |
| 990 | 4.3 | -3.8 | 0.7 | 10.7 | 0.595 | 0.107 |
| 1000 | 4.3 | -3.8 | 0.6 | 10.7 | 0.598 | 0.107 |
| 1010 | 4.3 | -3.8 | 0.7 | 10.7 | 0.602 | 0.103 |
| 1020 | 4.3 | -3.8 | 0.6 | 10.7 | 0.600 | 0.102 |
| 1030 | 4.3 | -3.7 | 0.6 | 10.7 | 0.605 | 0.102 |
| 1040 | 4.4 | -3.7 | 0.6 | 10.7 | 0.610 | 0.101 |
| 1050 | 4.2 | -3.7 | 0.6 | 10.7 | 0.610 | 0.098 |
| 1060 | 4.2 | -3.7 | 0.6 | 10.7 | 0.622 | 0.097 |
| 1070 | 4.3 | -3.7 | 0.6 | 10.7 | 0.607 | 0.101 |
| 1080 | 4.2 | -3.7 | 0.6 | 10.7 | 0.615 | 0.098 |

Appendix 4.3 (continue)

| Time | (Second) | MAXIM | MINIM | TV | SPLEU | REE | COMPL |
|---|---|---|---|---|---|---|---|
| 1090 | | 4.2 | -3.7 | 0.5 | 10.7 | 0.811 | 0.068 |
| 1100 | | 4.2 | -3.7 | 0.5 | 10.7 | 0.814 | 0.068 |
| 1110 | | 4.1 | -3.7 | 0.5 | 10.7 | 0.823 | 0.069 |
| 1120 | | 4.1 | -3.6 | 0.5 | 10.7 | 0.833 | 0.064 |
| 1130 | | 4.1 | -3.6 | 0.5 | 10.7 | 0.838 | 0.064 |
| 1140 | | 4.1 | -3.6 | 0.5 | 10.7 | 0.840 | 0.107 |
| 1150 | | 4.1 | -3.6 | 0.5 | 10.7 | 0.838 | 0.090 |
| 1160 | | 4.0 | -3.5 | 0.5 | 10.7 | 0.850 | 0.087 |
| 1170 | | 4.1 | -3.6 | 0.5 | 10.7 | 0.847 | 0.090 |
| 1180 | | 4.0 | -3.5 | 0.5 | 10.7 | 0.863 | 0.089 |
| 1190 | | 4.0 | -3.5 | 0.5 | 10.7 | 0.849 | 0.105 |
| 1200 | | 4.0 | -3.5 | 0.5 | 10.7 | 0.856 | 0.091 |
| 1210 | | 4.0 | -3.5 | 0.5 | 10.7 | 0.865 | 0.089 |
| 1220 | | 3.8 | -3.4 | 0.5 | 10.7 | 0.692 | 0.084 |
| 1230 | | 3.8 | -3.4 | 0.5 | 10.7 | 0.695 | 0.086 |
| 1240 | | 3.8 | -3.3 | 0.5 | 10.7 | 0.706 | 0.084 |
| 1250 | | 3.9 | -3.4 | 0.5 | 10.7 | 0.705 | 0.093 |
| 1260 | | 3.7 | -3.3 | 0.5 | 10.7 | 0.702 | 0.085 |
| 1270 | | 3.7 | -3.3 | 0.5 | 10.7 | 0.722 | 0.084 |
| 1280 | | 3.7 | -3.3 | 0.5 | 10.7 | 0.716 | 0.087 |
| 1290 | | 3.7 | -3.3 | 0.5 | 10.7 | 0.721 | 0.083 |
| 1300 | | 3.7 | -3.2 | 0.5 | 10.7 | 0.734 | 0.080 |
| 1310 | | 3.8 | -3.3 | 0.5 | 10.7 | 0.708 | 0.095 |
| 1320 | | 3.7 | -3.2 | 0.5 | 10.7 | 0.728 | 0.081 |
| 1330 | | 3.7 | -3.2 | 0.5 | 10.7 | 0.729 | 0.082 |
| 1340 | | 3.7 | -3.2 | 0.5 | 10.7 | 0.729 | 0.081 |
| 1350 | | 3.7 | -3.2 | 0.5 | 10.7 | 0.729 | 0.081 |
| 1360 | | 3.7 | -3.2 | 0.5 | 10.7 | 0.740 | 0.079 |
| 1370 | | 3.7 | -3.1 | 0.5 | 10.7 | 0.737 | 0.080 |
| 1380 | | 3.6 | -3.2 | 0.5 | 10.7 | 0.717 | 0.083 |
| 1390 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.738 | 0.079 |
| 1400 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.760 | 0.078 |
| 1410 | | 3.6 | -3.1 | 0.5 | 10.8 | 0.753 | 0.078 |
| 1420 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.766 | 0.077 |
| 1430 | | 3.8 | -3.2 | 0.5 | 10.7 | 0.728 | 0.092 |
| 1440 | | 3.7 | -3.2 | 0.5 | 10.7 | 0.742 | 0.091 |
| 1450 | | 3.6 | -3.1 | 0.5 | 10.8 | 0.762 | 0.077 |
| 1460 | | 3.6 | -3.1 | 0.5 | 10.8 | 0.759 | 0.078 |
| 1470 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.759 | 0.078 |
| 1480 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.760 | 0.077 |
| 1490 | | 3.6 | -3.1 | 0.5 | 10.8 | 0.771 | 0.087 |
| 1500 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.780 | 0.086 |
| 1510 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.796 | 0.076 |
| 1520 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.780 | 0.077 |
| 1530 | | 3.4 | -3.0 | 0.5 | 10.8 | 0.782 | 0.075 |
| 1540 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.760 | 0.077 |
| 1550 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.762 | 0.087 |
| 1560 | | 3.6 | -3.1 | 0.5 | 10.7 | 0.776 | 0.086 |
| 1570 | | 3.5 | -3.0 | 0.5 | 10.7 | 0.776 | 0.075 |
| 1580 | | 3.5 | -3.0 | 0.5 | 10.7 | 0.791 | 0.074 |
| 1590 | | 3.4 | -3.0 | 0.4 | 10.7 | 0.802 | 0.073 |
| 1600 | | 3.4 | -3.0 | 0.5 | 10.7 | 0.810 | 0.073 |
| 1610 | | 3.4 | -3.0 | 0.5 | 10.7 | 0.817 | 0.082 |
| 1620 | | 3.4 | -3.0 | 0.5 | 10.8 | 0.818 | 0.072 |

Appendix 4.3 (continued)

| Time | (Second) | MAXIN | MAXE | TV | PPLED | PEE | COMPL |
|---|---|---|---|---|---|---|---|
| 1830 | | 3.4 | -2.9 | 0.4 | 10.7 | 0.878 | 0.071 |
| 1840 | | 3.3 | -2.9 | 0.5 | 10.7 | 0.878 | 0.071 |
| 1850 | | 3.4 | -2.9 | 0.5 | 10.8 | 0.845 | 0.070 |
| 1860 | | 3.3 | -2.9 | 0.4 | 10.7 | 0.861 | 0.069 |
| 1870 | | 3.4 | -3.0 | 0.5 | 10.8 | 0.827 | 0.081 |
| 1880 | | 3.3 | -2.9 | 0.4 | 10.8 | 0.878 | 0.071 |
| 1890 | | 3.3 | -2.9 | 0.4 | 10.8 | 0.845 | 0.071 |
| 1900 | | 3.3 | -2.9 | 0.4 | 10.7 | 0.831 | 0.070 |
| 1910 | | 3.2 | -2.8 | 0.4 | 10.8 | 0.850 | 0.070 |
| 1920 | | 3.3 | -2.9 | 0.4 | 10.8 | 0.831 | 0.069 |
| 1930 | | 3.3 | -2.9 | 0.4 | 10.7 | 0.861 | 0.077 |
| 1940 | | 3.2 | -2.8 | 0.4 | 10.8 | 0.867 | 0.067 |
| 1950 | | 3.2 | -2.9 | 0.4 | 10.8 | 0.862 | 0.067 |
| 1960 | | 3.2 | -2.8 | 0.4 | 10.8 | 0.867 | 0.077 |
| 1970 | | 3.2 | -2.9 | 0.4 | 10.8 | 0.867 | 0.066 |
| 1980 | | 3.2 | -2.8 | 0.4 | 10.8 | 0.882 | 0.067 |
| 1990 | | 3.3 | -3.0 | 0.4 | 10.8 | 0.824 | 0.080 |
| 2000 | | 3.2 | -2.9 | 0.4 | 10.8 | 0.846 | 0.069 |
| 2010 | | 3.2 | -2.9 | 0.4 | 10.7 | 0.855 | 0.067 |
| 2020 | | 3.2 | -2.8 | 0.4 | 10.7 | 0.865 | 0.078 |
| 2030 | | 3.2 | -2.8 | 0.4 | 10.8 | 0.864 | 0.068 |
| 2040 | | 3.1 | -2.8 | 0.4 | 10.7 | 0.873 | 0.065 |
| 2050 | | 3.1 | -2.8 | 0.4 | 10.8 | 0.905 | 0.077 |
| 2060 | | 3.1 | -2.7 | 0.4 | 10.7 | 0.911 | 0.047 |
| 2070 | | 3.1 | -2.7 | 0.4 | 10.7 | 0.918 | 0.063 |

Appendix 5 The Effect of Different Polypeptides on Post-Experimental Lung Weight: Ratio of Lung Mass / Body Mass (g/g)
( data for figure 16, page 65 )

| Polypeptides | •Ratio of Lung Mass / Body Mass (g/g) | |
|---|---|---|
| Saline-150µl | 0.00596 | ± 0.000025 |
| Poly-Glu-2.25mg/150µl | 0.0055 | ± 0.000214 |
| Poly-Lys-0.0225mg/150µl | 0.0056 | ± 0.000187 |
| Poly-Lys-0.225mg/150µl | 0.00874* | ±0.000277 |
| Poly-Lys-2.25mg/150µl | 0.0142* | ±0.00086 |
| Poly-Arg-0.0225mg/150µl | 0.00598 | ±0.000348 |
| Poly-Arg-0.225mg/150µl | 0.00864* | ±0.000509 |
| Poly-Arg-2.25mg/150µl | 0.015* | ±0.000775 |

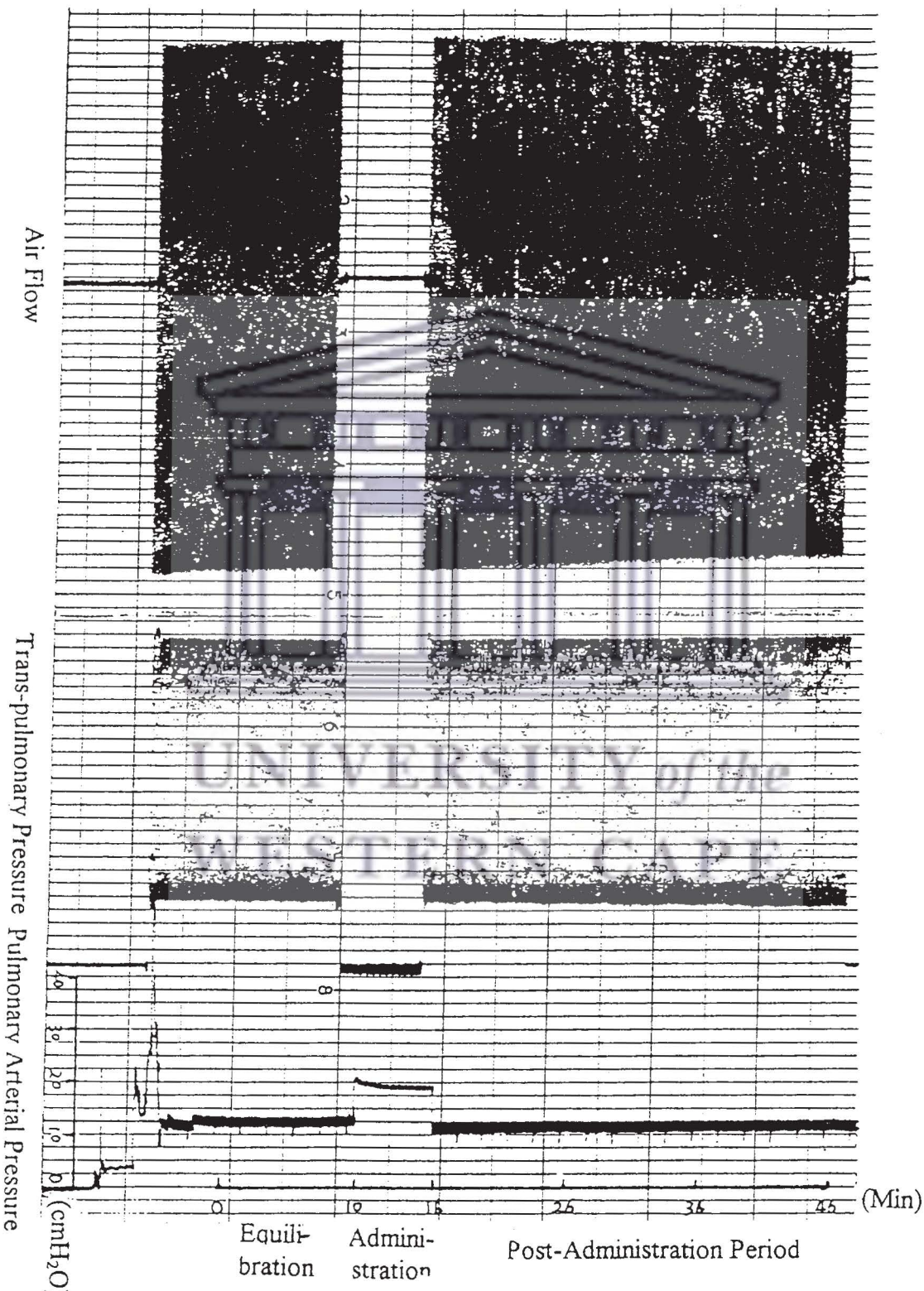
• Mean ± SE, n = 5; * Mann-Whitney U test, $P < 0.05$, alpha = 0.00714.

Appendix 6 Pulmonary Arterial Pressure pre-, during and after the Administration of Saline and the Different Polypeptides in the IPL

| Polypeptides | Pulmonary Arterial Pressure (in $cmH_2O$) during | | |
|---|---|---|---|
| | Equilibration Phase | Administration Phase | Post-Administration Phase |
| Saline-150µl | 13.4 ± 0.245 | 17.4 ± 0.245 | 13.5 ± 0.316 |
| Poly-Glu-2.25mg/150µl | 13.8 ± 0.374 | 17.6 ± 0.4 | 13.8 ± 0.374 |
| Poly-Lys-0.0225mg/150µl | 13.2 ± 0.339 | 17.4 ± 0.4 | 13.2 ±0.374 |
| Poly-Lys-0.225mg/150µl | 12.6 ± 0.245 | 17 ± 0.447 | 12.6 ±0.245 |
| Poly-Lys-2.25mg/150µl | 13.6 ± 0.4 | 17.2 ± 0.2 | 13.6 ± 0.4 |
| Poly-Arg-0.0225mg/150µl | 13.4 ± 0.225 | 17.4 ± 0.4 | 13.4 ±0.245 |
| Poly-Arg-0.225mg/150µl | 13.4 ± 0.4 | 18.2 ± 0.2 | 13.2 ±0.2 |
| Poly-Arg-2.25mg/150µl | 13.6 ± 0.245 | 17.8 ±0.374 | 13.8 ± 0.339 |

The pulmonary arterial pressure readings was recorded directly from the pressure meter and the Mean ± SE (n = 5) is given.

Appendix 7.1 Sample of Raw Data. Recorder Traces of Air Flow, Transpulmonary pressure and Pulmonary Arterial Pressure Changes after the Administration of Saline



Appendix 7.2 Sample of Raw Data. Recorder Traces of Air Flow, Transpulmonary pressure and Pulmonary Arterial Pressure Changes after the Administration of 0.225 mg Poly-Lysine