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The Isolated Perfused Lung

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The unique nonrespiratory functions of the lungs have become more apparent in recent years. The isolated perfused lung model offers many advantages over other methods for the study of pulmonary metabolism, xenobiotic disposition and the influence of interactions among agents of different physical forms. Detailed descriptions of the experimental preparation are elements in evaluating and comparing data from various sources but these are frequently neglected. A discussion and critique of the following elements are provided in this review in order to elucidate the typical problems one might encounter in evaluating data: perfusate type, perfusion method, construction materials, ventilation method, temperature control, surgical procedure, microbiological contamination and evaluation criteria of the preparation. Examples are given where the IPL method has been applied and suggestions are made for future research efforts.

The nonrespiratory capabilities of the pulmonary tissues have received increasing interest in recent years as the unique functions of the lungs have become more apparent. Because the respiratory tract is the main portal of entry and one of the first surfaces contacted by airborne contaminants, it is frequently the target organ for lesions produced by these agents. Thus, the pulmonary disposition of pollutants in the form of gases, vapors or aerosols may be important in their ultimate toxicity. The normal integrity of lungs may also be influenced by substances which enter the body by routes other than inhalation, for example by intravenous, intramuscular and transcutaneous routes, and are transported first to the pulmonary tissues by the circulatory system. This is a particular concern when agents are absorbed by the lymphatics which empty into the venous return perfusing the lungs (*1,2*).

It is apparent from an increasing number of studies that the lungs have the capacity to accumulate, bind and metabolize a variety of substances, both endogenous and exogenous. A large number of these studies have been reviewed by Niemeier (*3*), Dalbey (*4*), Fouts (*5*) and more recently by Roth (*6*).

As the understanding of metabolic and related processes at a molecular level becomes more complete, investigators are turning to complex systems in which the integration of such processes in an intact tissue may be studied. The technique of organ perfusion lies between the isolated organelle preparation, the tissue homogenate and slice, and the intact animal.

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There is presently no method that can be used to study pulmonary metabolic activity *in vivo* because of the influence of other organs. *In vivo* sampling, which assumes the ability to sample pulmonary arterial and venous concentrations of experimental chemicals, is a questionable method in xenobiotic studies (*7,8*). Whole animal preparations are useful for investigating pulmonary effects on vasoactive substances (*9*), but the technique's general application to metabolism of foreign compounds by the lung is very doubtful.

In contrast to other *in vitro* methods, cells in the isolated perfused lung (IPL) are maintained in their "normal" anatomical and physiological associations and are not fragmented or dispersed; therefore, transcellular transport and diffusion of agents are probably not altered. There are very few transected cells leaking their contents into the medium of the IPL, compared to tissue slicing methods, and there is no dilution of intracellular cofactors (especially when using whole blood) as occurs in homogenate or isolated organelle experiments. The isolated perfused lung preparation, in addition to metabolism studies, also offers the opportunity to investigate administration of multiple agents in different physical forms, the effectiveness of particle size and the distribution and binding of substances throughout the pulmonary system as mediated by the lungs composite metabolic machinery, i.e., pulmonary alveolar macrophage, tracheobronchial tissue, endothelial cells and alveolar tissue. As mentioned by Roth (*6*), evidence based on lung perfusion experiments performed in anesthetized patients, undergoing cardiac revascularization, strongly suggests that the isolated perfused lung technique adequately reflects the dynamic biochemical events occurring *in vivo*. Law et al. (*10*) compared the drug-metabolizing activities in microsomes prepared from perfused and nonperfused lungs

and found no significant differences in cytochrome P-450 content, NADPH-cytochrome reductase, benzphetamine demethylase, or benzyprene hydroxylase.

One of the more critical elements of IPL investigations is a detailed description of the experimental preparation, if the results of such studies are to be compared and repeated. Prior to the early 1970s, few published reports gave sufficient details on the methodology (*3,11,12*). These deficiencies included lack of detailed information on the apparatus, ventilation method, perfusion medium, operative procedure and the criteria by which the preparation could be evaluated. These items are of substantial importance in guiding the new investigator. Niemeier and Bingham (*11*) described a number of characteristics which they considered desirable for an IPL system in order to reproduce an accurate lung model in which "normal" physiological and biochemical conditions and variables are maintained to study xenobiotic metabolism, uptake, binding and excretion. Their model was developed for the rabbit lung and subsequently modified (*13*). Dalbey (*4*) and Dalbey and Bingham (*14*) further modified this system for use with rats and guinea pigs.

Because the details on methodology are rarely discussed and appear to have some bearing on the significance and utility of the model, the following summary and discussion was felt to have value in orienting new investigators to the field. The rationale for this approach is that the more recent IPL systems were specifically designed for xenobiotic metabolism studies with the intent of preserving the integrity of the lung over a period of several hours by maintaining "normal" physiological and biochemical functions as near to *in vivo* as possible.

Perfusate Types

Although a number of perfusates have been reported, undiluted (heparinized), autologous, whole blood is preferred (*15,16*) in order to avoid the homologous blood syndrome with pooled blood (*17–19*) or satisfy various factors, such as platelet dependency (*20*). Autologous whole blood is superior to other perfusates in delaying edema formation (*21–23*), which is one of the major problems with many IPL preparations. Rhoades (*24*) reported successful rat lung perfusions using KHB buffer supplemented with 6 wt-% bovine serum albumin and washed bovine red cells. Without red cells in the perfusate over one-half of the perfused lungs exhibited marked edema, i.e., weight gain over 5%. Rhoades remarked that washed cells in the medium appeared to maintain capillary patency. The use of undiluted whole blood is also based on the need for physiological blood gas tensions and pH, as well as availability of nominal concentrations of cofactors, endogenous substrates and trace metals. Ritchie et al. (*16*) considered undiluted whole blood to be the best perfusate from the physiological and biochemical point of view. Another advantage in the use of autologous whole blood may be the instance where maintenance of exogenous chemical(s) in the perfusate is desired to study interactive effects on metabolism, distribution, binding, etc., for example where the influence of chemical "X" administered by inhalation or diet for a defined period is investigated for its effects on chemical "Y." Thus, using autologous whole blood precludes the establishment of new equilibrium situations which would occur with altered media. Such use allows a more reliable estimate of the interactions to be studied without alterations in plasma and tissue levels of the agent.

Roth (*6*) and others, while accepting the divergence from physiological conditions, prefer artificial salt perfusion media supplemented with serum albumin, which prevents pulmonary edema. The former investigator expressed concern that whole blood components could bind and metabolize xenobiotics, lowering their effective free concentration and making kinetic studies difficult to interpret, especially in a recirculating system. Gillis and Iwasawa (*25*) eliminated serum albumin from their rabbit lung perfusion media and Roth (*6*) noted that this caused a major disadvantage and criticism of this system because of the slow flow rate (10 mL/min) requirement. He mentioned that higher flow rates in the absence of serum albumin would cause severe pulmonary edema. Since artificial salt solutions require serum albumin to prevent edema at physiological flow rates, the issue of binding by plasma proteins probably has no practical significance.

It should be emphasized that the use of whole blood more closely resembles the *in vivo* situation. Orton et al. (*26*) have stated that whole blood permits a better examination of lung-drug interactions, as binding of drug to plasma and red blood cells, proteins and lipids, etc., is part of the complex dynamic equilibrium occurring in the intact animal. Bingham et al. (*27*) indicated that metabolites of benzo(a)pyrene are distributed differently when comparing plasma to red cells. This observation reflects the importance of using whole blood, since distribution, absorption and excretion kinetics are important parameters in estimating the total disposition of a chemical. However, the influence of whole blood on overall metabolic rate and binding of xenobiotics should be routinely questioned. Its importance can readily be determined by simple incubation studies of the various components with the appropriate parent compound or metabolite.

Perfusion Methods

Recirculation of the perfusate is desirable because it eliminates the need to pool blood from donors and may be important in assessing slowly metabolized chemicals. In view of the fact that less than 60 mL of whole blood is necessary for the rabbit IPL (*11*) and 11 to 15 mL is needed for rats and guinea pigs (*14*), the preselected volume of medium to be recirculated can be small, but this may limit the duration of perfusion, depending on

the number of samples and the sample volume collected. The quantity of substrate required for a study is not a limiting factor, compared to "once-through" systems. An equilibrium is usually established between the perfusate and the pulmonary tissue since the relative volumes are fixed; however, increasing substrate concentrations of these limited volumes is possible with infusion or accelerated infusion techniques. Rates of uptake and metabolite production may be determined by serial analyses of the perfusate and minute concentrations of metabolites may be magnified by reducing the volume of perfusate or extending perfusion time.

Some disadvantages of the recirculation technique may include: accumulation of toxic metabolites of either endogenous or exogenous origin; accumulation of rate-limiting xenobiotic metabolite(s); exhaustion of substrates and other essential factors during perfusion; the possibility of damage to the perfusion medium over time with the use of a circulatory pump, e.g., hemolysis, frothing, denaturation of protein, crystal or particle formation as a result of drying.

The once-through perfusion technique has the advantage that a simpler system is required, i.e., the perfusate pump can be eliminated and a gravity type infusion used. However, there are many disadvantages with this technique. The design must be carefully considered, since a simple large reservoir is not an adequate substitute, unless it is designed so that large volume changes do not cause significant variations (± 2 cm H_2O) in hydrostatic pressure. A large volume of perfusate may be required, depending on the flow rate used and the duration of the experiment. It may also be necessary to simplify the medium because of cost or lack of available materials. Equilibrium may not be readily established with this technique, since the medium is constantly changing. This in itself may not be a disadvantage, especially in the instance where metabolite accumulation may be the rate-limiting factor. In the once-through technique it is inherent in the technique that the metabolic changes be established by determining the arteriovenous concentration differences which may be limited by the capabilities of the existing analytical method. In addition, the rate of flow through the pulmonary vasculature must be accurately determined on a continual basis in order to assess total substrate presented and total metabolite produced per unit of time. An accurate measurement may be difficult with smaller species where flow rates are very low.

Overall, the choice of the perfusion method is dependent on the experimental design. The recirculating perfusate method probably has advantages that far outweigh its disadvantages. Further discussion of the kinetics of both methods have been presented by Nagashima and Levy (*28*).

Constant blood pressure and flow are both desirable for a stable physiological preparation and the latter is essential for kinetic studies. The Niemeier-Bingham system utilizes a method employing a constant pressure reservoir and electronic level sensors where blood pressure is maintained at 23 cm H_2O. Hydrostatic pressures much greater than this may cause rapid onset of pulmonary edema; however, systematic studies have not been reported.

Increasing pulmonary vascular resistance is a common and serious problem in IPL preparations (*29*) and it is usually accompanied by massive edema (*4*). The Niemeier-Bingham model is not routinely plagued by this response (*13*) due to administration of epinephrine and therefore blood flow remains essentially constant throughout the experiment. Typical blood flows range from 160 to 240 mL/min in the rabbit preparations, depending on the individual preparation. Blood flow ranges obtained in rats and guinea pigs are 30 to 40 mL/min and 40 to 65 mL/min, respectively, with guinea pig preparations usually being more stable (*4*). Others have reported flow rates in these ranges, but usually lower flow rates are observed. One of the most critical preventive measures of sudden irreversible edema and/or complete cessation of flow is the avoidance of air bubbles in the perfusion circuit (*3,25,30*). At relatively high flow rates, such as cited above, there is a higher risk of this occurring, but because of the rapidity of the response, the likelihood of detecting air bubble formation is low. Therefore, it is extremely important when using the higher flow rates to meticulously eliminate all possible sources of bubble formation. The likely sources are at tubing connections, valves, cannulae and air/ perfusate interfaces.

The advantages of constant pressure perfusion are: it maintains automated control of the flow rate and therefore eliminates the need for pressure monitoring equipment and subsequent frequent and careful adjustments in pump rates; it provides the ability to administer and sample agents from the pulmonary arterial perfusate with relative ease; it allows direct determination of relative pulmonary vascular resistance through convenient flow measurements, i.e., determining changes in calibrated pumping rates.

Materials

Chemically inert equipment, i.e., all siliconized glass, to prevent platelet adhesion (*17*) and augment perfusate recovery, should be used as much as possible. Materials such as metal, plastics and rubber are kept to a minimum or eliminated to avoid the possibility of toxic reaction and/or chemical interactions from leached materials such as platicizers, and/or absorption of the compound by these materials (*31–33*). Orton and coworkers (*26*) reported that in experiments with aniline and propazine, it was necessary to subtract absorption of the chemical to the tubing at each time point. Similarly they reported a 40% adsorption rate of prometone to silicon rubber tubing, which prevented further investigation of the chemical. Law et al. (*10*) mentioned that tygon tubing was found to absorb large amounts of parathion and therefore, glass tubing was substituted. Blase and Loomis (*30*) reported rapid

adsorption of carbaryl by silicon rubber and polyethylene tubing. This was remedied by replacing with like components of glass and Teflon except for a small piece of silicon rubber tubing necessary for the peristaltic pump. They replaced the silicon rubber tubing after each perfusion. Niemeier (*3*) noted a negligible rate of ^{14}C-B(a)P adsorption (0.02% in 2.5 hr) to silicon rubber tubing. It is obvious from these limited data that careful attention must be given to both the choice of materials used to construct a system and the compatability of the chemical to be studied with the various components of the system.

Ventilation Methods

Ventilation of the lung can be accomplished by two modes, subatmospheric ("negative") pressure or "positive" pressure inflation. Commercially available small animal respirators can be used for either mode. Positive pressure ventilation requires connection of the respirator directly to the lung. Subatmospheric ventilation involves using a reverse connected respirator to cycle subatmospheric pressures in a container in which the lung is suspended. It is important in either case to filter and humidify the ventilating gas. The latter step prevents water loss, tissue drying and hemoconcentration. Preheating the gas to 37°C also prevents excessive heat loss from the preparation and water condensation in the ventilation tubing.

Subatmospheric ventilation is preferred, since positive pressure ventilation may lead to destruction of lung architecture by overinflation and subsequent edema and progressive atelectasis (*34–36*). Overinflation by subatmospheric ventilation combats edema and atelectasis by preserving functional residual volume and allows a periodic "sighing" maneuver which is known to regenerate surfactant coatings and further help to minimize atelectasis (*37*).

With the use of the tracheal valve (*13*) much better control of extratracheal dead air space is possible than with other systems. This valve thus minimizes rebreathing of the ventilating gas (and contaminants) and permits intratracheal administration during perfusion with relative ease. Routine spirometry and collection of expired gases during perfusion are other advantages of using this valve system.

The composition of ventilating gas is perhaps the most divergent of all variables used in IPL techniques. The composition ranges from air to various combinations of O_2, N_2 and CO_2 with air and 95% O_2–5% CO_2 being the most popular (*3*). Very limited information is available in the literature which addresses the effects of various gas compositions on metabolism in the IPL (*6,24,33*). One might expect that 95% O_2–5% CO_2 is preferred with artificial media and air is preferred when using blood, but evidence for this is not usually found in the literature. A precautionary note, however, is warranted based upon the evidence presented by Fisher (*38*), where serotonin uptake in the isolated perfused lung was markedly diminished by exposure to 100% oxygen at one atmosphere. It is not known whether other active transport processes are similarly affected by high partial pressures of oxygen. Obviously further work is needed in this area for which the primary goal should be to define gas mixtures that produce stable biochemical and physiological conditions, i.e., acid base balance, stable pH, etc., with various perfusates.

In the Niemeier-Bingham system, acid production, presumably due to lactic acid and CO_2 (carbonic acid) (*38*) by erythrocytes and lung tissue causes the pH to fall quite rapidly. Na bicarbonate (0.3 mEq/hr) in glucose (30 mg/hr) solution is added at a rate of 0.3 mL/hr to counterbalance this effect and actually create slightly more alkaline conditions. In the most recent refinement of Niemeier-Bingham technique the pH is controlled between 7.38 and 7.42 by mixing CO_2 with air through an automatic feedback system consisting of a continuous monitoring pH meter whose output signal is fed to a voltage sensing relay with dual setpoints, which, in turn, controls a switch for an electrical solenoid valve regulating influx of CO_2.

Temperature Control

Another design variable of IPL systems is the means of temperature control of the system. There are two major techniques generally reported in the literature: the environmental box and water-jacketed components or variations thereof. Most systems use a temperature of 37°C (*3*). Systems designed by Rhoades (*24*), O'Neil and Tierney (*39*), and Tucker and Shertzer (*40*) are examples of environmental chambers. Niemeier and Bingham (*11*), Orton et al. (*26*), McGovren et al. (*41*) and Dalbey and Bingham (*14*) are examples of systems which utilize water-jacketed components. Gillis and Iwasawa (*25*) employed a variation of the latter in that the lung chamber and perfusate lines were inserted into a constant temperature water bath. The major consideration in the use of these designs is the convenience of the method. For instance if intratracheal administration is necessary, opening of the environmental box to permit these injections would allow large variations in temperature and humidity. In general, the environmental box is cumbersome (*26*) and offers more constraints than water-jacketed components in the manipulation of the system.

Operative Procedure

Another variation among IPL systems is the manner in which anesthesia and surgery are performed. The types of "anesthesia" range from cervical dislocation (*10,11,30*) to administration of CO_2 (*40,42*), halothane (*39*), urethane (*43*) or pentobarbital sodium (*26,44*). It would suffice to mention that careful attention must be given to the type used in relation to the chemical being studied, since interactions, i.e., competitive enzyme binding sites, depletion of high energy intermediates,

changes in pulmonary vascular tone affecting perfusion rate, etc., may influence the "normal" metabolic outcome.

Some investigators (*39,42*) perfuse and ventilate the lungs during surgery. Some (*30,45*) perfuse the vasculature *in situ* to remove blood and prepare the lungs for the artificial perfusate, while others (*11,26,41*) neither ventilate nor perfuse during the surgical procedure. Although no systematic investigations have been reported which assess the effects of ischemia on the isolated perfused lung system, the latter system of nonperfusion/nonventilation during surgery would be expected to cause the most severe problems. However, as mentioned above, Law et al. (*10*), using the Niemeier-Bingham system as modified by Orton et al. (*26*) found no significant differences in drug-metabolizing activities of microsomes prepared from nonperfused lungs compared with those from lungs perfused up to 4 hr. In addition, Orton et al. (*26*) reported that no detectable differences were found during light or electron microscopic examination between perfused and nonperfused lungs and that no signs of degeneration or inflammatory reactions were observed.

Therefore, for the sake of simplicity, it appears that ventilation and/or perfusion during surgery are unnecessary to preserve the functional integrity of the pulmonary system. It should be noted that with the Niemeier-Bingham system, as well as with a few other systems, the vasculature can be readily cleared of blood by initially perfusing with artificial media, making it unnecessary to perfuse the vasculature during surgery.

Microbiological Contamination

One potential problem commonly overlooked is the possibility of bacterial contamination and subsequent concern for extraneous metabolism due to this contamination. Since current reports state that the isolated perfused lung is viable for up to 5 hr, there is a real concern that log-phase bacterial growth can occur in the perfusate, especially the artificial types which are not usually reported to be handled aseptically. This is not only a potential problem with recirculating type systems but may also occur with "once-through" preparation. This author is not suggesting the use of antibacterial agents in the perfusate. This may only further confuse interpretation of metabolic studies. However, extreme care should be used, as with any tissue or organ culturing system, to preclude bacterial contamination. This care should be exercised in the surgical phase, throughout perfusion and during clean-up of the system, i.e., sterile technique should be adhered to as much as possible. It is also evident that animals should be discarded if there are obvious signs of infection.

Evaluation Criteria

Although few investigators have reported on the biochemical and physiological stability of their preparation, measurements of these parameters are essential to defining the preparation. As mentioned previously (*3,11,13,14*), some of the more obvious methods which have been used in judging the stability of a preparation as compared to *in vivo* values were: perfusate flow, pH, spirometry measurements, net weight gain (edema), hematocrit, glucose uptake, microscopy, perfusate gas tensions, and various biochemical measurements (*11*). More sophisticated evaluations have been reported by Law et al. (*10*) comparing xenobiotic enzyme activity of the preparation before and after perfusion. Perhaps other evaluations of stability, such as the assessment of ventilation/perfusion ratios, regional alveolar/arterial PO_2 gradients, dynamic compliance, small airway resistance, etc., should be considered for their applicability as additional measures.

In general, a systematic investigation is needed to determine which of these measures more accurately reflects the stability of a preparation as they are related to the IPL metabolic activity. This is perhaps more important when studying slowly metabolized chemicals. The ultimate choice of methods used should give consideration to ease, simplicity, and reliability of the method plus the fact that simultaneous sampling and analyses must be performed on the chemical being studied. For these reasons most investigators have relied mostly on constant perfusate flow rate, stable pH and weight gain as indicators of a stable preparation.

Applications

The final area of discussion will focus on the possible manipulations and pertubations which are possible with the IPL system. They include *in vivo* treatment prior to perfusion, such as with classical enzyme-inducing agents (*13,14,27,42,43,46*) or with active transport or metabolic inhibitors (*30,47*). Pretreatment of animals using an inhalation exposure regimen has also been reported, for example using *n*-dodecane (*48,49*), sulfur dioxide (*50,51*) and cigarette smoke (*46*). The effects of subchronic treatment by intratracheal injection of various particulates have also been investigated (*52*).

During perfusion chemicals to be studied can be administered by intratracheal administration or by injection into the perfusate. However, more complex administrations have been attempted. Warshawsky et al. (*50,51*) reported concomitant intratracheal injection of benzo(a)pyrene absorbed onto a particulate with simultaneous inhalation exposure to sulfur dioxide gas. McGovren et al. (*41*) administered tobacco smoke to the isolated perfused lung by attaching a cigarette directly to the tracheal cannula and lighting it. Reports of other aerosol studies using the IPL are not evident, but Schanker (*53*) commented on the utility of the IPL to study drug absorption from the lung. Large gaps in our knowledge of aerosol deposition and disposition exist and the isolated perfused lung technique may be an important tool that can be utilized in this area. A major obstacle to this effort in the past has been the technol-

ogy for the routine generation and characterization of vapors and aerosols on a scale small enough to be practical for IPL investigations. Some excellent recent resources for commencing these types of investigations are now available (*54,55*).

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