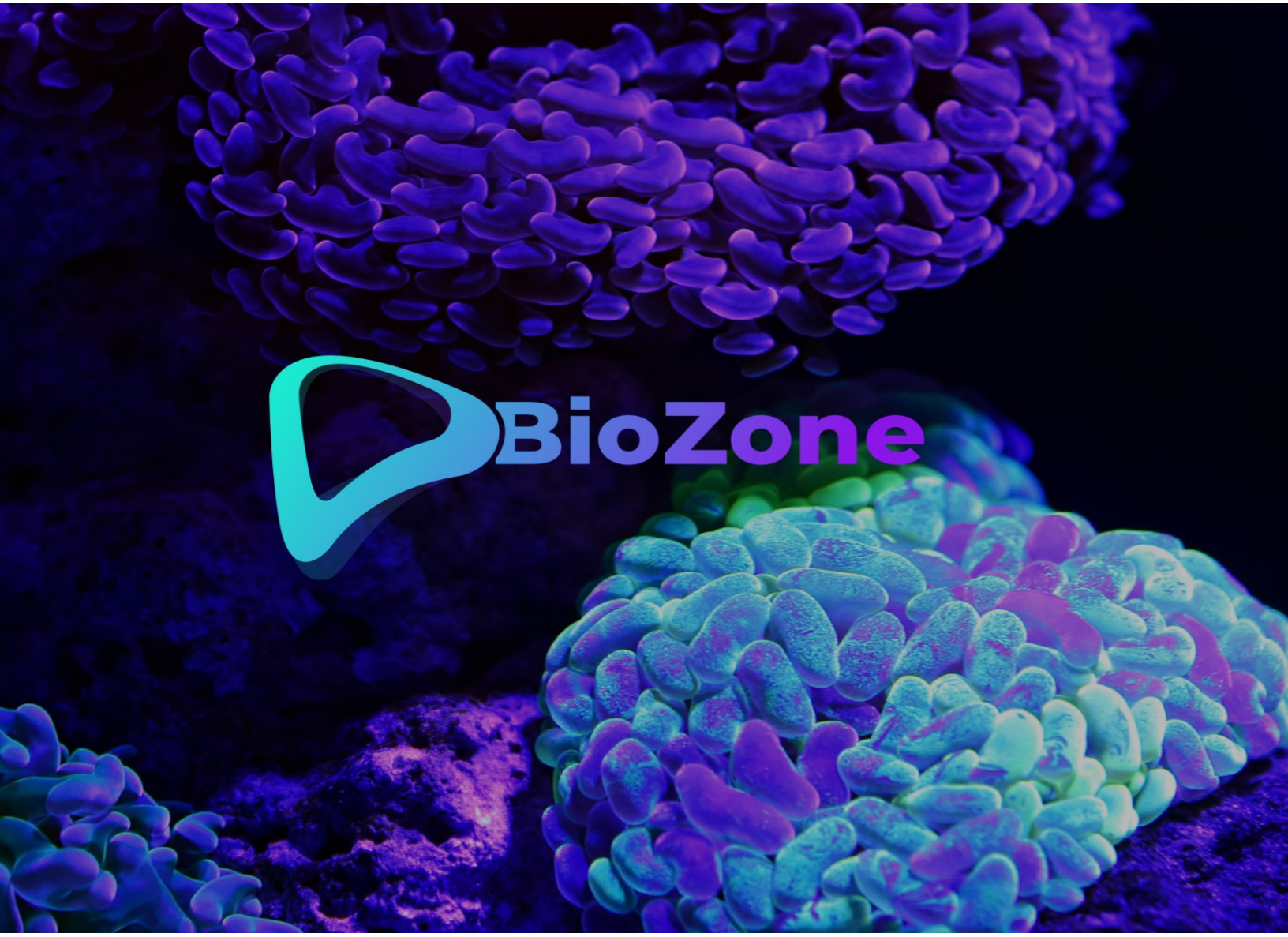
BIOZONE TECHNICAL DISCUSSIONS



BIO-ATMOSPHERE DRUG DELIVERY SYSTEM

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NOTE: The individual reading the following technical review of the literature should rely on a considerable background expertise and understanding of Pathology, Physiology, Biochemistry, Pharmacology, and Immunology.

Novel technologies must rely on science and innovation to provide lifesaving solutions to critically ill patients as well as the prevention thereof. The BioZone System of technologies will focus on introducing corporeal therapies to reduce the consequences of excessive 'Inflammation' on vital organs. Vertu Realities is developing such corporeal therapies that target the effector cells that drive systemic inflammation, causing direct tissue damage and secreting a range of pro-inflammatory cytokines that initiate and propagate imbalanced immune responses. It will be made evident in the following discussions that by intervention and mitigation of these inflammatory and immune responses a broad spectrum of disease processes can be positively affected or alleviated.

I. PULMONARY DRUG DELIVERY; A PROMISING FUTURE

A. The anatomy and physiology of the Respiratory System

The human lung consists of 5 lobules and 10 bronchopulmonary segments. Arranged adjacent to each segment are lung lobules composed of 3–5 terminal bronchioles. Each bronchiole supplies the smallest structural unit of the lung, the acinus, which consists of alveolar ducts, alveolar sacs, and alveoli. Alveolar epithelial type I cells represent the principal cell type lining the surface of the alveoli. The major functions of these cells, which cover 93% of the alveolar space, are to provide a surface for gas exchange and to serve as a permeability barrier. Alveolar epithelial type II cells have a much smaller surface area per cell and they represent 16% of the total cells in the lung. They play a basic role in synthesis, secretion and recycling of surface-active material (lung surfactant). The alveolar blood barrier in its simplest form consist of a single epithelial cell, a basement membrane, and a single endothelial cell. While this morphologic arrangement readily facilitates the exchange, it can still represent a major barrier to large molecules. Before entering the systemic circulation, solutes must traverse a thin layer of fluid, the epithelial lining fluid. This layer tends to collect at the corners of the alveoli and is covered by an attenuated layer of surfactant. Unlike the larger airways, the alveolar region is lined with a surface-active layer consisting of phospholipids (mainly phosphatidylcholine and phosphatidylglycerol) and several key apoproteins. The surfactant lining fluid plays an important role in maintaining alveolar fluid homeostasis and permeability and participates in various defense mechanisms. Recent studies suggest that the surfactant may slow down diffusion out of the alveoli.

The respiratory airways, from the upper airways to the terminal bronchioles, are lined with a viscoelastic, gel-like mucus layer 0.5–5.0 mm thick. The secretion lining consists of two layers: a fluid layer of low viscosity, which surrounds the cilia (periciliary fluid layer), and a more viscous layer on top, the mucus. The mucus is a protective layer that consists of a complex mixture of glycoprotein's released primarily by the goblet cells and local glands. The mucus blanket removes inhaled particles from the airways by entrapment and mucociliary transport at a rate that depends on viscosity and elasticity. The lung tissue is highly vascularized, which makes pulmonary targeting difficult because of fast absorption of most drugs (especially lipophilic and low molecular weight drugs)

The lung itself consists of around 300 million alveoli which are the smallest air sac components of the lung. Each alveolus individually is surrounded by a plexus of blood vessels which end on end would amount to be about 6 kilometers long. These vessels are so small that they total only 5.4% of the lung volume. The total alveoli measured as surface area for a human adult lung is around 80 to 100 square meters or the size of either a racquetball court or a tennis court according to the changes in volume during inhalation and exhalation. Approximately 5 to 10 liters of air is moved through the tracheobronchial tree every minute with a subject at rest. Diagram (1) is a representation of the physiological structures of the lungs with conducting and respiratory zones.

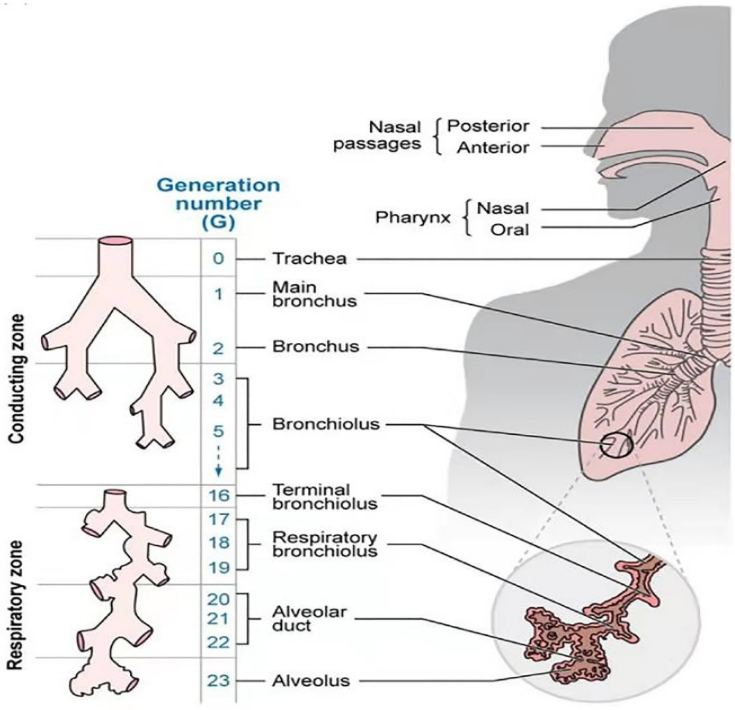


Diagram (1) The physiological structure of the lungs with conducting and respiratory zones. The conducting zone (G0–G16) comprises of the trachea, bronchi, bronchioles and terminal bronchioles, which is responsible for conducting

air to the respiratory regions of the lungs. The respiratory bronchioles, alveolar ducts and alveolar sacs constitute the respiratory zone (G17–G23), which facilitates the gas exchange between the airspaces and blood capillaries.

Blood is oxygenated in the lungs through respiration where the oxygen molecules travel from the air through the lung anatomy and then through the alveolocapillary membrane interface into the blood.

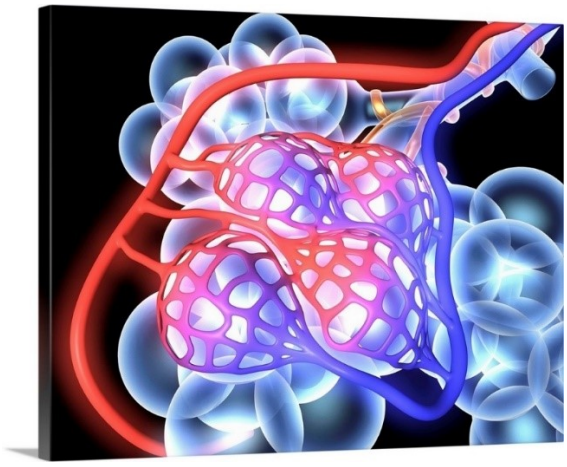


Diagram (2) is an artist rendering of this alveolocapillary relationship.

In medical terminology oxygenation is commonly used to refer to oxygen saturation. Again, in medical common use oxygen saturation (SO2) is commonly referred to as “sats”, which is the measurement of the percentage of hemoglobin binding sites in the bloodstream occupied by oxygen. At low partial pressures of oxygen, most hemoglobin is deoxygenated. At around 90% oxygen saturation increases according to an oxygen-hemoglobin dissociation curve and approaches 100% at partial pressures of > 10 kPa.

Healthy individuals at sea level usually exhibit oxygen saturation values between 97% and 99%. An arterial oxygen saturation value below 90% causes hypoxemia. Hypoxemia due to a low arterial oxygen saturation is indicated by cyanosis, but oxygen saturation does not directly reflect tissue oxygenation. The affinity of hemoglobin to oxygen may impair or enhance oxygen release at the tissue level. Oxygen is more readily released to the tissues when the PH is decreased, body temperature is increased, arterial partial pressure of carbon dioxide is increased, and 2,3 DPG levels (a byproduct of glucose metabolism also found in stored blood products) are increased. When the hemoglobin has greater affinity for oxygen, less is available to the tissues, Conditions such as an increased blood PH, decreased temperature, decreased partial pressure of carbon dioxide, and decreased 2,3 –DPG will increase oxygen binding to the hemoglobin and thus limit its release to the

tissue. The relationship of these variables is expressed in a sloped graph called the oxygen- hemoglobin dissociation curve (see diagram 3).

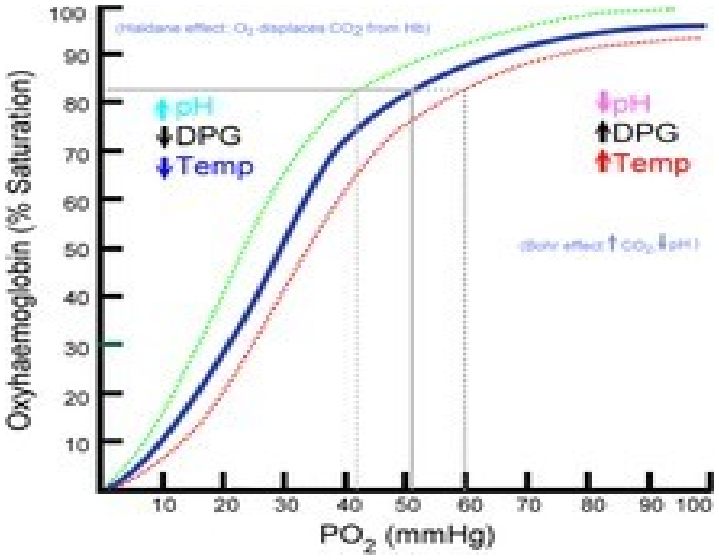


Diagram (3); Oxygen-Hemoglobin Dissociation Curve

Assessing a patient's need for oxygen is the most essential element to life; no human life thrives in the absence of oxygen and this is why we establish medical algorithms for serious medical presentations, assessing the airway and breathing before any other parameter is inspected. Although a pulse oximeter is used to monitor oxygenation, it cannot determine if the metabolism of oxygen or the amount being used by the patient. For this purpose, it is necessary to also measure carbon dioxide (CO2) levels. It is possible that it can also be used to detect abnormalities in ventilation. However, the use of a pulse oximeter to detect hypoventilation is impaired with the use of supplemental oxygen, as it is only when patients breathe room air that abnormalities in respiratory function can be detected reliably with its use. Therefore, the routine administration of supplemental oxygen may be unwarranted if the patient is able to maintain adequate oxygenation in room air, since it can result in hypoventilation going undetected.

Relevant to oxygenation is the discussion of the circulatory system and the dynamics negative pressure conditions exert on the microcirculation system. The human circulatory system is an organ system delivering nutrients, gases, hormones, blood cells, etc. to and from cells in the body wherein this delivery system helps fight diseases and stabilizes body temperature and PH to maintain homeostasis. Humans have a closed cardiovascular system meaning that the blood never leaves the network of arteries, veins and capillaries. The lymphatic system, on the other hand, is an open system. Two types of fluids move through the circulatory system; blood and lymph. The blood, heart, and blood vessels form the

cardiovascular system. The lymph, lymph nodes, and lymph vessels form the lymphatic system. The cardiovascular system and the lymphatic system collectively make up the circulatory system.

The main components of the human cardiovascular system are the heart and blood vessels. It includes the pulmonary circulation and the systemic circulation that provides oxygenated blood to the tissues. Blood is oxygenated in the lungs through a gas exchange process whereby the CO2 produced from metabolism and energy production is eliminated from the lungs into the atmosphere and oxygen from the atmosphere is absorbed into the pulmonary system which is in turn is bound to the hemoglobin molecules to be circulated from the heart to every cell in the body. Oxygen and nutrients carried by the blood have to diffuse across blood vessel layers to enter the interstitial fluid which carries the oxygen and nutrients to the target cells. Carbon dioxide and metabolic waste products have to diffuse in the opposite direction to be eliminated from the body.

About 98.5% of the oxygen in a sample of arterial blood in a healthy human breathing air at sea level pressure is chemically combined with hemoglobin molecules. About 1.5% is physically dissolved in the other blood components and not combined with the hemoglobin.

The microcirculation is a term used to describe the small vessels in the vasculature which are embedded within organs and are responsible for the distribution of blood within tissues; as opposed to larger vessels in the macrocirculation which transport blood to and from the organs. The vessels on the arterial side of the microcirculation are called the arterioles, which are well innervated, are surrounded by smooth muscle cells, and are 10-100 μm in diameter. Arterioles carry the blood to the capillaries, which are not innervated, have no smooth muscle, and are about 5-8 μm in diameter. Blood flows out of the capillaries into the venules, which have little smooth muscle and are 10-200 μm. The blood flows from venules into the veins. In addition to these blood vessels, the microcirculation also includes lymphatic capillaries and collecting ducts. The main functions of the microcirculation include the regulation of 1. blood flow and tissue perfusion 2. blood pressure, 3. tissue fluid (swelling or edema), 4. delivery of oxygen and other nutrients and removal of CO_2 and other metabolic waste products, and 5. body temperature. The microcirculation also has an important role in inflammation.

Most vessels of the microcirculation are lined by flattened cells, the endothelium, and many are surrounded by contractile cells the smooth muscle or pericytes. The endothelium provides a smooth surface for the flow of blood and regulates the movement of water and dissolved materials in the plasma between the blood and the tissues. The endothelium also produce molecules that discourage the blood

from clotting unless there is a leak. The smooth muscle cells can contract and decrease the size of the arterioles and thereby regulate blood flow and blood pressure.

Flow dynamics can be integrated into capillary blood flow and the Hagen-Poiseuille equation predicts the flow of blood through vessels being proportional to the diameter and length of the vessels in microcirculation. The Starling equation describes the roles of hydrostatic and osmotic forces (Starling Forces) in the movement of fluid across capillary endothelium (See diagram (4)).

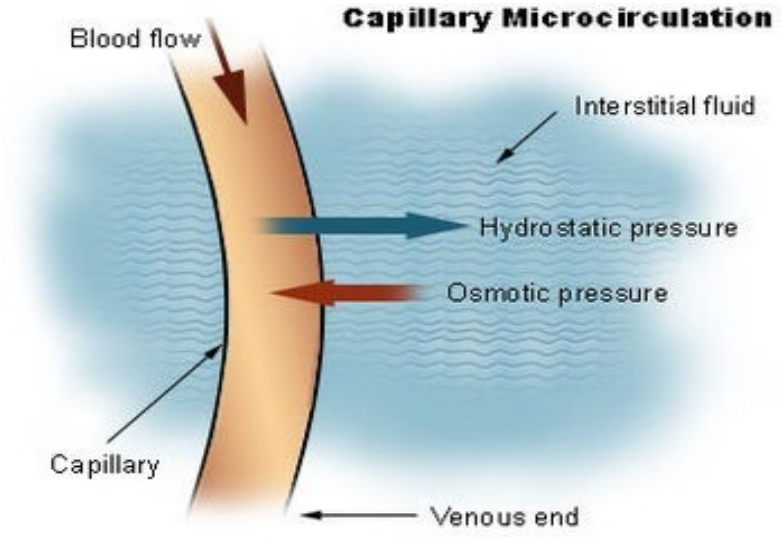


Diagram (4); Capillary Microcirculation

Additional studies have revealed that cutaneous circulation is also regulated by cardiopulmonary baroreceptors and negative pressure applied to cutaneous surfaces have been shown to enhance blood flow and improve wound healing. Intermittent variable negative pressure gradients can be utilized to further enhance cutaneous microcirculation and the larger the variable gradient the greater the negative pressure effects. For instance, varying an intermittent negative pressure gradient from -10 mm Hg to -75 mm Hg had a greater effect on microcirculation enhancement than varying a smaller gradient such as going from -45 mm Hg to -65 mm Hg. Measuring the increased microcirculation effect of a constant negative pressure environment and the intermittent varying negative pressure gradient effect can be calculated and measured with laser doppler velocimetry and plethysmography.

Visual verification of cutaneous microcirculation enhancement secondary to negative pressure exposure is readily demonstrated by applying a suction cup to an area of skin and noting the vasoactive result.

B. Pulmonary Drug Delivery

The controlled release of drugs for pulmonary delivery is a research field which has been so far rather unexploited but is currently becoming increasingly attractive. The development of controlled release formulations for inhalable drugs has been widely investigated for several years. However, up to now, sustained release formulations for pulmonary delivery have not been marketed yet in spite of the increasing interest. There are many advantages to developing sustained release formulations for pulmonary drug delivery, including reduced dosing frequency, improved patient compliance and reduction in side effects. The reduction of the dosing frequency is of great concern for a number of pulmonary disorders including asthma and chronic obstructive pulmonary disease (COPD). In particular, long acting β2- adrenergic receptor agonists with glucocorticoids used for the relief of asthma- and COPD-related bronchospasm have a plasma half-life that constrain the patient to an administration of the drug every 5.5 hours. A controlled release formulation leading to a prolonged duration of action of more than 8 hours would prevent nocturnal exacerbation in bronchial asthma. Controlled release formulations are widely used in oral or parenteral formulations but have not been established for pulmonary applications.

Current research approaches include the use of liposomes, micro- and nanosuspensions and dry powder formulations. In this purpose the liposomes have been the most extensively investigated carriers. They can be prepared with lung endogenous phospholipids as surfactants, with a wide range of size and are able to incorporate both hydro and lipophilic drugs. Liposomes proved to be able to impart a sustained release profile to the incorporated substances but they also present some disadvantages, i.e., a high production cost, a relative instability during storage and nebulization that can lead to their disruption and to the premature loss of entrapped substances. Therefore, liposomal dry powder formulations are currently getting more attractive.

Polymeric microspheres have also been successfully tested in vitro as well as in vivo as sustained release drug delivery system. They are more physicochemical stable than liposomes, both in vitro and in vivo, allowing thus a slower release of encapsulated drugs. Their main disadvantage is that their safety still remains uncertain. It was showed that pulmonary administration of PLA microspheres to rabbits led to histological damages assessed in terms of pulmonary hemorrhage, eosinophilia and neutrophil infiltration. Inflammation can, however, be avoided using large porous particles. To date, the commercial use of such products is thus difficult. For similar reasons it is also difficult to aerosolize a particle suspension in a way to ensure a constant delivered dose to the lung. Therefore, dry powder

formulations have attracted attention. The formulation typically contains structural components of the particle as well as agents allowing the release of the drug over an extended period of time. These include lipids, proteins, sugars or synthetic polymers such as poly (vinyl alcohol) or polyesters. In particular, PLGA has been widely used, as it is considered biodegradable and weakly toxic.

Nevertheless, achieving substantial bioavailability of proteins and macromolecules by this route has remained a challenge, chiefly due to poor absorption across the epithelium. The lungs are relatively impermeable to most drugs when formulated without an absorption enhancer/promoter and many novel absorption promoters have been tested for enhancing the systemic availability of drugs from the lungs. Various protease inhibitors, surfactants, lipids, polymers and agents from other classes have been tested for their efficacy in improving the systemic availability of protein and macromolecular drugs after pulmonary administration. Several small, nonpolar drugs cross the respiratory mucosa passively and act systemically (i.e. nicotine and marijuana).

The lungs possess many favorable characteristics of an alternate drug delivery site including a low intrinsic enzymatic activity, large absorptive surface area (100 m^2), extensive vasculature, thin layer alveolar epithelium (0.1–0.2 μm) and short distance of air–blood exchange passage. The high surface area and high permeability of the lungs make them an ideal site for rapid systemic delivery of macromolecules and small-molecule drugs; however, the formulation of the drug is of crucial importance in getting the drug to the right place for optimal absorption. Small molecules are absorbed more rapidly through the lungs than through the gastrointestinal tract, with higher bioavailability and reduced first-pass metabolism by enzymes. The lungs are significantly permeable to many peptides and proteins, with the rate of absorption decreasing with increasing molecular mass.

Pulmonary drug delivery has therefore attracted tremendous scientific and biomedical interest in recent years and has progressed considerably within the context of local treatment for lung diseases, by virtue of enhanced local targeting and reduced systemic side effects with the administration of minute drug dosages. Furthermore, with the high surface area and permeability of the lung, the 21st century has seen a paradigm shift to inhaled therapy for systemic use. But the pulmonary tract tends to be considered as very promising and attractive route for the administration of active substances intended to treat local pulmonary e.g., asthma, chronic obstructive pulmonary disease (COPD), microbial infections) as well as systemic diseases (e.g., diabetes).

Studies on the delivery of drugs to or via the respiratory tract have been carried out in the recent 25 years. This route can offer considerable advantages and disadvantages over other drug administration ways as listed below,

Advantages

- Provides local action within the respiratory tract
- Provides rapid drug action
- Provides reduced dose
- Allows for a reduction in systemic side-effects It can be employed as an alternative route to drug interaction when two or more medications are used concurrently
- Reduces extracellular enzyme levels compared to GI tract due to the large alveolar surface area
- Reduces evasion of first pass hepatic metabolism by absorbed drug
- Offers the potential for pulmonary administration of systemically active materials

Disadvantages

- The duration of activity is often short-lived due to the rapid removal of drug from the lungs or due to drug metabolism.
- Necessitates frequent dosing.

Recent progress within biotechnology has generated a group of novel protein and peptide drugs to which administration to the respiratory tract, to obtain systemic delivery seems advantageous compared to parenteral or gastrointestinal administration (tablets, capsules etc.). The low metabolic activity in the lungs allows systemic delivery without liver passage hence lung is an attractive environment for biomolecules, which are highly susceptible to enzymatic

degradation in the gastrointestinal tract (ventricle and guts) as well as hepatic degradation (first pass metabolism).

C. Uptake of inhaled drug after inhalation therapy

As stated, there are several advantages in delivering drugs to the lungs including a noninvasive method of delivery; the surface area of the lung is between 80 m2 and 140 m2, which is about half the area of a tennis court. In addition, in most pulmonary regions, the thickness of the alveolar epithelium is only between 0.1 μm and 0.2 μm. The total distance between epithelial surface and blood in the alveolar area is between 0.5 μm and 1.0 μm which are much less than in the bronchial system (distance between mucus surface and blood: 30 μm–40 μm). Thus, it appears that pharmaceuticals after deep inhalation and deposition in the peripheral alveolar region of the lung can be rapidly absorbed. Pulmonary delivery therefore has the advantage, compared to nasal delivery, that it is possible to obtain a sufficiently high absorption without the need of enhancers.

On the other hand, the human lung has different defense mechanisms to prevent aerosol particles penetrating into the deep lung. Primarily, the oropharyngeal region and the bronchial tree are excellent filters to eliminate aerosol particles from the inhaled air and particles deposited on ciliated epithelium are subject to mucociliary transport to the gastrointestinal tract. Therefore, to deliver a drug into the deep lung, one has to surmount these filters. However, even after deposition in the alveolar region of the lung, a number of mechanisms inhibit the absorption of inhaled pharmaceuticals. There are a number of absorption barriers (mucus layer, alveolar lining fluid layer, macrophages and other cells, alveolar epithelium and basement membrane) which act to varying extents by inhibiting drug permeation into the circulation, there exists competing cellular uptake pathways (particle phagocytosis by macrophages), and of course proteolytic degradation can limit the amount of intact drug available for absorption.

The function of these barriers can be impaired by very different substances and consequently the absorption of drugs can be increased, for example, by the use of absorbance enhancers (cyclodextrins, detergents and bile acids). Furthermore, proteolytic degradation can be inhibited by protease inhibitors (nafamostat mesilate and aprotinin, for example) and phagocytosis by macrophages reduced by packaging of substances into porous particles. In principle, absorption kinetics of inhaled substances depend on their molecular weight (small molecules are more rapidly absorbed than larger ones), pH-value, electrical charge, solubility and stability of the inhaled substance.

The other target regions within the lung for inhalable drugs are the large and small bronchial airways. Different pulmonary diseases are located in these parts of the respiratory tract. The most relevant are asthma, chronic obstructive pulmonary disease (COPD) and bronchial tumors. To treat these diseases locally, one has to deliver the drugs specifically to this region.

Parameters determining particle deposition in deep lung

Different biophysical parameters determine regional drug deposition in the human lungs:

- Aerodynamic particle behavior (size, density, hygroscopicity, shape, electrical charge).

- Breathing pattern of the patients (flow rate, ventilation volume, end-inspiratory breath holding).

- Time of aerosol pulse injection into the breathing cycle.

- Anatomy of the respiratory tract.

Of these factors, aerosol particle size and size distribution are the most influential on aerosol deposition. The aerodynamic particle diameter (AD) is the diameter of a sphere with a density of 1 g/cm3 that has the same aerodynamic behavior as the particle which shall be characterized. In that way, aerosol particles with different density and shape can be characterized depending on their aerodynamic properties.

Aerodynamic particle behavior

The size of the particles is a critical factor affecting the site of their deposition since it determines operating mechanisms and extent of penetration into the lungs. The aerodynamic diameter is also defined as the equivalent diameter of a spherical particle of unit density having the same settling velocity from an air stream as the particle in question. Thus, particles that have higher than unit density will have actual diameters smaller than their AD. Conversely, particles with smaller than unit density will have geometric diameters larger than their AD.

Aerosol size distributions may be characterized as practically monodisperse (uniform sizes) or polydisperse (non-uniform sizes). The upper airways (nose,

mouth, larynx, and pharynx) and the branching anatomy of the tracheobronchial tree act as a series of filters for inhaled particles. Thus, aerosol particles bigger than 100 μm generally do not enter the respiratory tract and are trapped in the naso/oropharynx. The particles must be very fine, for example having an aerodynamic diameter of less than 10 μm. Particles having aerodynamic diameters greater than 10 μm are likely to impact the walls of the throat and generally do not reach the lung. Particles having aerodynamic diameters in the range of 5 μm to 0.5 μm will generally be deposited in the respiratory bronchioles whereas smaller particles having aerodynamic diameters in the range of 2 to 0.05 μm are likely to be deposited in the alveoli. Particles in the ambient air are transported by different physical mechanisms. The relevant mechanisms for therapeutic aerosols are diffusion by Brownian motion (particles in the size range of 0.5 μm), which is the random motion of particles suspended in a medium such as a liquid or gas, and impaction (size range >3 μm).

Mechanism of drug deposition:

The mechanisms by which particles deposit in the respiratory tract includes impaction (inertial deposition), sedimentation (gravitational deposition), Brownian diffusion, interception, and electrostatic precipitation. The relative contribution of each depends on the characteristics of the inhaled particles, as well as on breathing patterns and respiratory tract anatomy. All mechanisms act simultaneously, but the first two mechanisms are most important for large-particle deposition within the airways (1 mm, AD, 10 mm). Diffusion, however, is the main determinant of deposition of smaller particles in peripheral regions of the lung. Impaction occurs when a particle's momentum prevents it from changing course in an area where there is a change in the direction of bulk air flow. It is the main deposition mechanism in the upper airways, and at or near bronchial branching points. The probability of impaction increases with increasing air velocity, breathing frequency, and particle size. Sedimentation results when the gravitational force acting on a particle overcomes the total force of the air resistance. Inspired particles will then fall out of the air stream at a constant rate. This is an important mechanism in small airways having low air velocity. The probability of sedimentation is proportional to residence time in the airway and to particle size and decreases with increasing breathing rate. Diffusion occurs when the collision of gas molecules with small aerosol particles exerts discrete non-uniform pressures at the particles' surfaces, resulting in random Brownian motion. The effectiveness of Brownian motion in depositing particles is inversely proportional to particle diameters of those particles, 0.5 μm, and is important in bronchioles, alveoli, and at bronchial airway bifurcations. Molecule-size particles may deposit by diffusion in the upper respiratory tract, trachea, and larger bronchi.

Respiratory patterns

The pattern of respiration during aerosol exposure influences regional deposition, since breathing volume and frequency determine the mean flow rates in each region of the respiratory tract, which, in turn, influence the effectiveness of each deposition mechanism. Turbulence tends to enhance particle deposition, the degree of potentiating depending on the particle size. Rapid breathing is often associated with increased deposition of larger particles in the upper respiratory tract, while slow, steady inhalation increases the number of particles that penetrate to the peripheral parts of the lungs. Slow breathing, with or without breath-holding, showed a broad maximum deposition in the ciliated airways (tracheobronchial region). The pulmonary maximum occurred between 1.5 μm and 2.5 μm with breath holding and between 2.5 μm and 4μm without breath-holding. Rapid inhalation showed similar trends: the tracheo-bronchial region maximum falls and shifts to between 3 μm and 6 μm. Pulmonary deposition sharpens and occurs between 1.5 μm and 2 μm with breath-holding, and between 2 μm and 3 μm without breath-holding. When the above considerations are taken into account, the ideal scenario for aerosol would be:

- Aerosol AD smaller than 5 μm, to minimize oropharyngeal deposition
- Slow, steady inhalation and
- A period of breath-holding on completion of inhalation.

Pulmonary clearance

The primary function of the pulmonary defensive response to inhaled particles is to keep the respiratory surfaces of the alveoli clean and available for respiration. The elimination of particles deposited in the lower respiratory tract serves an important defense mechanism to prevent potentially adverse interactions of aerosols with lung cells. Insoluble particulates are cleared by several pathways, which are only partially understood. These pathways are known to be impaired in certain diseases and are thought to depend on the nature of the administered material. Swallowing, expectoration, and coughing constitute the first sequence of clearance mechanisms operating in the naso/oropharynx and tracheobronchial tree. A major clearance mechanism for inhaled particulate matter deposited in the conducting airways is the mucociliary escalator, whereas uptake by alveolar macrophage predominates in the alveolar region. In addition to these pathways, soluble particles can also be cleared by dissolution with subsequent absorption from the lower airways. The rate

of particle clearance from these regions differs significantly and its prolongation can have serious consequences, causing lung diseases from the toxic effects of inhaled compounds. It is now well recognized that the lungs are a site for the uptake, accumulation, and/or metabolism of numerous endogenous or exogenous compounds. All metabolizing enzymes found in the liver are also found in the lung, although in smaller amounts. The rate at which a drug is cleared and absorbed from the respiratory tract depends on the dynamic interaction of several factors, predominantly:

- The mucociliary clearance rate
- Site of deposition along the airways
- Biopharmaceutical factors (particulates vs. drug in solution)
- Drug release rate
- The physicochemical properties of the drug, such as molecular weight, partition coefficient, and charge.

Mucociliary clearance

Mucociliary clearance is a physiologic function of the respiratory tract to clear locally produced debris, excessive secretions, or unwanted inhaled particles. It consists of ciliated epithelial cells reaching from the naso/oropharynx and the upper tracheobronchial region down to the most peripheral terminal bronchioles. Beating of the cilia, together with mucus secreted by the goblet cells, contributes to an efficient clearance mechanism. For normal mucociliary clearance to occur it is necessary that the epithelial cells are intact, the ciliary activity and the rheology of mucus are normal, and that the depth and chemical composition of the periciliary fluid layer is optimal. Thus, the mucociliary escalator can be impaired by altering the volume of mucus secretion, the mucus viscosity and elasticity, or the ciliary beat frequency. Mucociliary clearance is known to be impaired in smokers, in patients with chronic bronchitis, and in acute asthmatics. Certain diseases have the opposite effect that of enhancing clearance rates.

D. Novel protein and peptide drugs

Recent progress within biotechnology has generated a group of novel protein and peptide drugs to which administration to the respiratory tract, to obtain systemic delivery seems advantageous compared to parenteral or gastrointestinal administration (tablets, capsules etc.). For example, the low metabolic activity in the lungs allows systemic delivery without liver passage hence lung is an attractive environment for biomolecules, which are highly susceptible to enzymatic degradation in the gastrointestinal tract (ventricle and guts) as well as hepatic degradation (first pass metabolism). However, the respiratory system in itself restricts the entrance of particulate matter by various means: geometry of the airways and clearance mechanisms of the lungs. Consequently, inhalation particles have to be aerodynamically optimized to reach absorption sites in the alveolar epithelium.

Recently, advances in drug engineering and recombinant DNA technology have led to the production of numerous therapeutic protein/peptide drugs. These drugs are either enzymatically unstable or impermeable through the gastrointestinal tract and, therefore, require injection. The pulmonary route has generated considerable interest as a valid, noninvasive, systemic route of drug administration. A major obstacle to the widespread use of pulmonary drug delivery is the relative impermeability of the lungs to many of these peptides/macromolecular drugs when they are administered without an absorption enhancer/promoter.

However, the mechanism of action of these absorption enhancers could be due to an irreversible, distortion of the alveolar epithelial cell layer, which could potentially make the lungs susceptible to the entry of exogenous allergens and dust particles inhaled during respiration. Thus, the use of absorption promoters in pulmonary drug delivery has generated safety concerns regarding possible long-term effects. In some cases, however, the permeabilization of the lungs has been demonstrated to be reversible with short-term use of the absorption enhancers in pulmonary protein delivery. The bulk of these agents have been divided into one of the following major classifications: (I) protease inhibitor (II) surface-active agents (III) liposomes and phospholipids (IV) cyclodextrins (V) miscellaneous agents.

Protease inhibitors

The concept of including enzyme inhibitors in pulmonary drug formulations has been researched extensively. The mechanism of action of protein absorption enhancement by protease inhibitors is thought to be due to a reduction in the proteolytic activity of various enzymes, which are responsible for degrading susceptible molecules such as insulin. The amount of absorption enhancement will typically rely on what enzyme the protease inhibitor inhibits (serine inhibitor, aminopeptidase, for example).

Surface-active agents

The mechanisms of action by which the surface-active agents increase the alveolar-capillary transfer of solutes are not entirely characterized. The mechanism of these agents may involve an increase in transcellular transport via an interaction and/or fluidization of the cell membrane which subsequently makes it more permeable, a modulation of tight junctions and increase in paracellular permeability, or some combination of both.

Liposomes and phospholipids

The use of liposomes has been suggested to provide sustained pulmonary release for various drugs. However, liposomes and phospholipids have also been investigated for the systemic absorption of different proteins after intratracheal delivery. The mechanism of absorption enhancement by liposomes may be attributed to the presence of surfactants on alveolar surface. The lung surfactants consist primarily of dipalmitoyl phosphatidyl choline and low amounts of surfactant.

Cyclodextrins

Cyclodextrins have been the topic of intense research for their applications in drug delivery over the past decade. These species are cyclic oligomers of glucose and form inclusion complexes with drugs whose molecules can fit into the lipophilic cavities of the cyclodextrin molecule. The potential of cyclodextrins, especially the methylated cyclodextrins, as absorption enhancers of proteins has been demonstrated in one case for luteinizing hormone-releasing agonist.

Miscellaneous agents

Lanthanide ions (Ln^{3+}) have been employed as protein absorption promoters in pulmonary drug delivery. The absorption of porcine zinc insulin preadministered or coadministered with Ln^{3+} ions from the lung was investigated by means of an in situ pulmonary absorption experiment in male wistar rats. The relative bioavailability of insulin after intratracheal administration with $CeCl_3$ and $GdCl_3$ was 57.9% and 59.5%, respectively. $LaCl_3$ demonstrated weak enhancement in pulmonary absorption.

E. Micro and nanoparticles

The introduction of micro- and nanoparticles in medicine aims to optimize treatment by improving the bioavailability and blood longevity of the selected therapeutic and by increasing its accumulation at the biological target (thus

reducing side effects). Considering intravenous administration, which is the most common for such formulations, the main limitations are (i) the high accumulation of particles inside organs of the reticuloendothelial system (RES is the liver, spleen, and lungs) and (ii) the poor extravasation of particles, which also depends by the size of the vector itself. While the former reduces the circulation time of particles, the latter makes it very difficult to reach the target tissue. RES clearance and poor extravasation are commonly accepted as the two most critical among the so-called "biological barriers," with serum opsonins and degrading enzymes (which can favor particle clearance), and the intracellular endo-lysosomal system (tasked with degrading a certain number of internalized objects) completing the list.

The accumulation of microparticles by the RES depends on the presence of resident macrophages inside the vascular lumen. These cells, which are part of the immune system, continuously sense the surrounding area in order to catch and clear particulates of different origins (cell debris, pathogens). The biological origin of this process is part of regular immune surveillance conducted by phagocytic cells, both inside organs and within the circulatory system, and represents a fundamental process in immunity. Once microparticles are injected systemically, circulation will carry them past the organs of the RES and subsequently in close proximity to macrophages which may recognize these objects as potential intruders to be removed. It is important to note that the liver, spleen, and lungs are highly vascularized organs, and the presence of macrophages is considerable. As such, the mammalian body is programmed to eliminate particles via the RES organs and this represents a problem when designing microparticles for therapy or diagnosis.

Moreover, recent studies have demonstrated that physico-chemical particle factors and biological phenomena mediate macrophage-clearance of blood-borne particulates. This is mainly dependent on (i) the biomolecules (including opsonins) adsorbed to the particles and (ii) their stiffness. Both parameters influenced the design of micro- and nanoparticles to decrease clearance by macrophages. PEGylation of particles, for example, is an efficient modification that reduces opsonin adsorption on the particle surface.

The possibility to modulate particle stiffness and design particularly soft particles is another means to minimize RES clearance of particles, and this approach was consciously chosen as a way to prolong DPN circulation time following systemic injection. The rationale to use a very soft particle to inhibit particle clearance by macrophages is supported by the mechanisms by which old red blood cells (RBCs) are eliminated from the blood. While aging, RBC becomes more rigid over time. Their removal from circulation is a process moderated by resident macrophages of the spleen, and only occurs when their stiffness allows phagocytic cells to correctly

orient and engulf them. Flexible RBCs cannot be internalized since macrophages cannot correctly anchor their surface. In line with this reasoning, recent literature provided evidence that deformable DPNs are less subject to phagocytosis in RES organs.

A possible explanation of this behavior is that soft particles are able to avoid being embraced by phagocytic cells. Deforming their structure upon contact, soft DPNs are less phagocytized and thus able to navigate the circulatory system longer and eventually depositing a larger number at the original biological target.

Due to their size in relation to "pore size" in the vascular endothelium, microparticles are poor at extravasating and thus require a rational and considered strategy to optimize delivery. This, of course, can be tailored to the specific pathology or by a series of modifications and functionalization to the particle surface. However, some general considerations need to be made. If we consider it to be impossible for a micrometric particle to extravasate, the only process which can be optimized is particle accumulation in the vasculature at the diseased site. This process strictly depends on (i) particle margination and adhesion to the walls of the diseased tissue vessels and (ii) the pathology of those vessels (e.g., tumor vasculature is profoundly different from healthy vessels). It is important to note that, despite the importance of particle surface functionalization, taking into consideration particle geometry to enhance marginalization in circulatory flow can completely change the game. Thus, DPNs are designed to optimize margination, and discoids have a greater tendency to navigate the vessels (in presence of blood) at their margins compared with spherical particles.

When a particle formulation is injected into the blood flow, particle trajectory is governed by the interplay between hydrodynamic forces, near-wall lift force, and adhesive interactions of particle ligands receptors on the endothelium. Typically, blood flow is characterized by erythrocytes migrating to the low-shear zone in the channel core by virtue of their deformability. Concomitantly, the leukocytes and platelets marginate laterally towards the vessel wall due to low deformability and their collisions with the erythrocytes. This phenomenon has inspired the design of polymeric drug delivery formulations, since increasing particle interaction with the vessel wall can promote drug transport past the endothelium into the tissue. The ideal formulation would then be capable of laterally drifting towards the walls to interact with the vessel endothelium but also be able to deform to avoid non-specific clearance.

F. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues

Mucus is a viscoelastic and adhesive gel that protects the lung airways, gastrointestinal (GI) tract, vagina, eye and other mucosal surfaces. Most foreign particulates, including conventional particle-based drug delivery systems, are efficiently trapped in human mucus layers by steric obstruction and/or adhesion. Trapped particles are typically removed from the mucosal tissue within seconds to a few hours depending on anatomical location, thereby strongly limiting the duration of sustained drug delivery locally. A number of debilitating diseases could be treated more effectively and with fewer side effects if drugs and genes could be more efficiently delivered to the underlying mucosal tissues in a controlled manner. This review first describes the tenacious mucus barrier properties that have precluded the efficient penetration of therapeutic particles. We will then review the design and development of new mucus-penetrating particles that may avoid rapid mucus clearance mechanisms, and thereby provide targeted or sustained drug delivery for localized therapies in mucosal tissues.

Nanoparticle systems can be engineered to possess a number of desirable features for therapy, including:

(i) sustained and controlled release of drugs locally,
(ii) deep tissue penetration due to the nano-metric size,
(iii) cellular uptake and sub-cellular trafficking, and
(iv) protection of cargo therapeutics at both extracellular and intracellular levels.

In order to avoid rapid mucus clearance mechanism and/or reach the underlying epithelia, nanoparticles must quickly traverse at least the outermost layers of the mucus barrier (that is cleared most rapidly). Mucus layer thickness depends strongly on anatomical site and can range from less than 1 micron up to several hundred microns. Until recently, nanoparticles were thought incapable of efficiently penetrating mucus layers.

In order to penetrate mucus, synthetic nanoparticles must avoid adhesion to mucin fibers and be small enough to avoid significant steric inhibition by the dense fiber mesh. Recently, it was demonstrated that nanoparticles as large as 500 nm, if sufficiently coated with a muco-inert polymer, can rapidly traverse physiological human mucus with diffusivities as high as only 4-fold reduced compared to their rates in pure water. This finding suggests that it is possible to engineer nanoparticles that overcome the mucus barrier. Combined with a suitably tailored drug release profile, these “mucus-penetrating particle” (MPP) systems offer the

prospect of sustained drug delivery at mucosal surfaces and, thus, provide hope for improved efficacy and reduced side effects for a wide range of therapeutics. The generation of MPP loaded with nucleic acids may also greatly enhance the efficacy of this critical family of therapeutic agents.

The human body naturally produces microparticles under pathologic conditions (e.g., cancer, endothelial alterations, inflammation), resulting from membrane blebbing as part of cell apoptosis. Artificial microparticles (e.g., DPNs) can mitigate this risk by minimizing protein adsorption (through their materials) and by tuning the particle materials properties (i.e., particle deformability and size). By controlling these physico-chemical properties, occlusions of narrow, distal vessels can be avoided as a particle size, surface corona, and deformability control macrophage uptake and aggregation.

Vascular diseases

Vascular diseases consist of a number of different conditions that affect central, peripheral, venous, and arterial blood flow and are due to alteration of the endothelium. The endothelium is the layer of cells that coats the inner lining of the blood and lymphatic vessels, allowing fluid, cell, and nutrient transport around the body. Moreover, it is crucial for innate and acquired immunity and for the regulation of vasomotor tone. On a molecular level, the endothelium regulates the transport of fluids and solutes between the blood and tissues. Although the endothelium is semipermeable and in the basal state, there is a continuous passage of substances through the vessel walls, and permeability can be regulated by specific external signals.

Damage to the endothelium or homeostatic dysregulation, due to and guided by inflammation and hypoxia, is observed as a result of tumors, heart attack, or other pathological conditions. Such damage often causes a deleterious increase in endothelial permeability. Furthermore, endothelial damage and a pro-inflammatory environment increase the adhesion of circulating immune cells such as monocytes, macrophages, neutrophils, leukocytes. With the progression of inflammation, the infiltration of immune cells through the vessel walls, along with the release of matrix metalloproteinase and other proteolytic molecules, destabilize endothelial integrity, and increasing vessel permeability. DPNs are potentially useful tools for the treatment of vascular diseases due to their ability to marginate towards the walls of the blood vessels, interact with damaged vasculature, and for their good average circulation half-life (around 24 h).

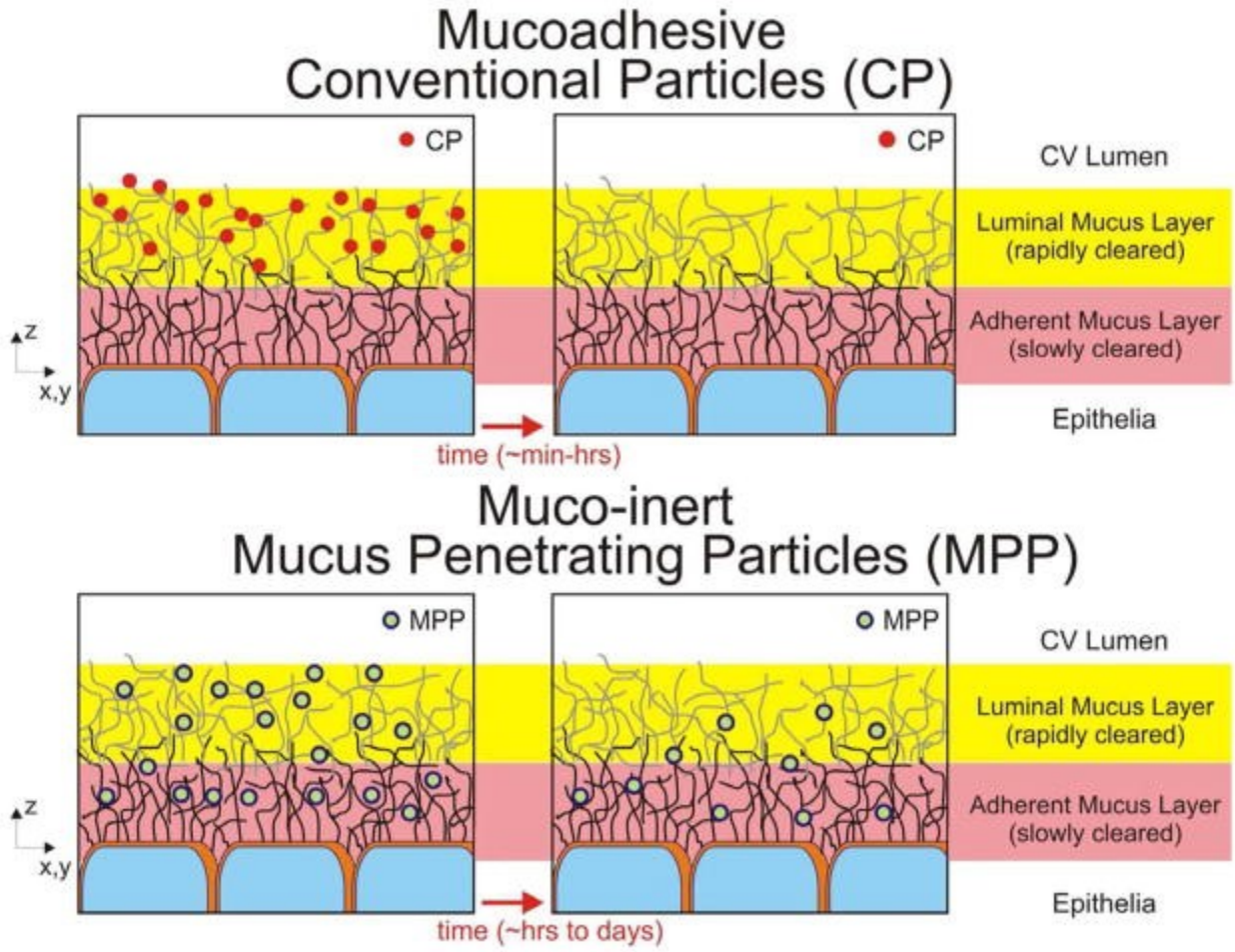
G. Typical fate of nanoparticles in mucus-covered tissues & conventional strategies for enhancing residence time

When administered to various mucosal tissues, conventional nanoparticles are likely to be trapped by mucus via steric or adhesive forces and rapidly eliminated via mucociliary clearance. To overcome the short transit time, research has largely centered on minimizing the fraction of therapeutics undergoing direct transit and fecal elimination by improving their association to mucus. This phenomenon, known as mucoadhesion, is widely defined as the ability of polymeric systems to adhere to the mucus layer. Mucoadhesion slows the particle transit time through the GI tract to the time scale of mucus renewal, thereby enhancing drug absorption. The design of mucoadhesive drug delivery systems is currently the predominant approach to improving mucosal delivery of therapeutics. Many researchers have sought to enhance the mucoadhesion of particles in order to improve their retention at mucosal surfaces. To maximize association with mucus, a variety of mucoadhesive drug delivery systems have been engineered, driven by various interaction forces between mucus and nanoparticles, including hydrogen bonding, van der Waals interactions, polymer chain interpenetration, hydrophobic forces, and electrostatic/ionic interactions. Electrostatic interaction is one of the most exploited forms of mucoadhesion, as exemplified by chitosan, a cationic polymer obtained from deacetylation of chitin, for a variety of oral and nasal drug delivery applications. Jubeh et al. concluded that cationic liposomes adhered to healthy colonic rat mucosa at rates 3-fold greater than neutral or anionic liposomes. Particles synthesized from common biomaterials, such as polyethylene glycol, polycarbophil and carbopol (derivatives of polyacrylic acid), poly(methacrylates), and poly(sebacic acid) may achieve mucoadhesion via hydrogen bonding, polymer entanglements with mucins, hydrophobic interactions, or a combination of these mechanisms.

Although mucoadhesion is a promising approach to increase the bioavailability of drugs delivered via mucosal tissues, important fundamental limitations of this approach exist. Since mucoadhesive systems are bound to the mucus layer through interactions with mucin fibers, the transit time of these systems is determined by the physiological turnover time of the mucus layer.

For oral delivery, considering the intestinal mucin turnover time is between 50–270 minutes, mucoadhesive particles are not expected to adhere to mucus for more than 4–5 hours. Furthermore, as mucoadhesive systems efficiently adhere to mucus, they are largely incapable of penetrating across the mucus layer and entering the underlying epithelia. Thus, mucoadhesive systems are especially unsuitable for delivery of drug and gene molecules that require intracellular delivery. To overcome these issues, various attempts have been made to engineer

particles that adhere specifically to intestinal cells, as exemplified by the conjugation of tomato lectins to nanoparticles. However, these ligand-bound particles appear to have a limited capacity to diffuse through the mucus layer and, instead, undergo premature adsorption to mucus. Thus, these systems reach the enterocyte surface inefficiently at best, and are instead bound to mucus, transported through the GI tract, and eliminated in the feces in a similar fashion to other mucoadhesive systems. To overcome this problem and achieve longer residence time of particles at mucosal surfaces, the foremost requirement is to engineer particles that can efficiently cross the mucus barrier.



Lessons from nature: transport of viruses in mucus

To gain mechanistic insight and rationally engineer particles to cross mucus, we must take an alternative approach and looked to nature for guidance. Specifically, the focus on understanding the physicochemical properties (size and surface chemistry) that govern the rapid transport of specific viruses, which have evolved over thousands of years to infect mucosal tissues. An indirect implication of the highly immobilized nature of virus-sized polystyrene beads in mucus is that viral

particles possess a sufficiently large surface area to undergo polyvalent adhesive interactions with mucus. Nevertheless, many viruses are capable of diffusing in mucus as fast as in water.

To develop mucus-penetrating particles (MPP) we can mimic the essential surface properties of viruses that allow them to avoid mucoadhesion. A hypothesis first proposed by Cone and coworkers noted that many viruses, including poliovirus, Norwalk virus and human papilloma virus (HPV), are densely coated with both positively and negatively charged groups, leading to a densely charged yet net neutral surface. It can therefore be rationalized that the high density of surface charge creates a hydrophilic surface that minimizes hydrophobic entrapment to mucus. Furthermore, an equal density of positive and negative charges may facilitate efficient mucus transport by allowing the viruses to avoid electrostatic adhesive interactions. Some viruses thus appear well designed to penetrate mucus by simultaneously possessing a muco-inert surface to avoid mucoadhesion and a sufficiently small geometry to diffuse through the low viscosity pores in mucus without significant steric obstruction.

H. Coating particles with low molecular weight polyethylene glycol

A faithful mimic of the muco-inert viruses would involve developing synthetic particles with a similarly high density of both cationic and anionic surface charge groups. However, although techniques have been advanced to generate multiple alternating layers of oppositely charged coatings with thicknesses on the order of angstroms, the engineering of such densely charged yet neutral coatings onto the surfaces of synthetic particles remains exceedingly difficult.

Treatment of mucus with mucolytics agents may improve the penetration rates of drug and gene carrier particles. The use of mucolytics as an adjuvant to particle transport may be particularly important for diseases where mucus is abnormally viscoelastic, such as cystic fibrosis and chronic obstruction pulmonary disease (COPD).

The possibility of using polymer nanoparticles for controlled drug or gene delivery at mucosal sites over many hours to days is expected to lead to effective new therapeutics. However, no such product currently exists since conventional therapeutic particles cannot penetrate the human mucosal barrier, which rapidly clears trapped pathogens and particulates. The development of mucus-penetrating particles (MPP), by rendering the surfaces of particles non-mucoadhesive via lessons learned from viruses, provides a powerful strategy for overcoming the mucus barrier. In particular, the efficient transport of large MPP (200 to 500 nm)

through human mucus should strongly encourage the commercial development of new nanoparticle-based drug delivery systems for use in various mucosal surfaces, since drug delivery kinetics and loading efficiency are vastly improved as particle diameter increases. As such, MPP offers the prospect of sustained delivery of a variety of potentially important drugs to treat diseases at mucosal tissues.

In recent years, mucus-penetrating particles (MPPs) have become a competitive candidate due to their enhanced efficiency in drug delivery by permeating the mucus barrier. With a hydrophilic and electrically neutral (i.e., muco-inert) surface, as in the case of viruses, and a particle size smaller than the mucus pore size, MPPs can easily penetrate through the mucus layer by reducing the interaction between the particles and mucus, improving the local drug delivery to mucosal epithelial cells. Coating nanoparticles with poly(ethylene glycol) (PEG) of a high density and low molecular weight is one of the most widely used strategies for mucus penetration. However, PEGylation usually cannot enhance cellular uptake and stimulate the production of neutralizing antibodies

Neutralizing antibodies, administrated through intravenous infusion, have shown to be highly efficacious in treating mild and moderate COVID-19 caused by SARS-CoV-2 infection in the lung. However, antibodies do not transport across the plasma-lung barrier efficiently, and up to 100 mg/kg dose was used in human causing significant supply and cost burdens. Studies look to explore the feasibility of nebulized antibodies inhalation delivery as an alternative route. The study demonstrated that nebulized neutralizing antibody delivery though inhalation could be a more efficient and efficacious alternative approach for treating COVID-19 and other respiratory infectious diseases and warrants further evaluation in clinical studies.

The monoclonal antibody therapies have shown excellent efficacies and safety profiles in treating mild and moderate COVID-19 patients. However, SARS-CoV-2 tends to be concentrated in respiratory system such as lungs, with very little in the bloodstream. A therapeutic antibody administered by intravenous injection needs to cross the plasma-lung barrier to exert its efficacy. Large biomolecules such as monoclonal antibodies don’t transport through the plasma-lung barrier efficiently. As a result, dosages of up to 100 mg/kg antibodies were used to treat COVID-19 patients in clinical trials, which put significant pressure on treatment cost and antibody production, thus limiting the widespread use of mAbs in developing countries.

Alternatively, nanovehicles with an efficient propulsion provide a new concept for enhancing the ability to overcome the mucus barrier. Nanovehicles are devices that can convert different forms of energy or fuel into kinetic energy and are

increasingly used in drug delivery, catalysis, environmental monitoring, and biosensing. Generally, nanovehicles include self-propelled catalytic nanomotors and fuel-free nanorobots powered by biocompatible external energy, such as light, electricity, sound, magnetism, and chemistry energies.

In particular, magnetically driven nanovehicles can be remotely actuated in living organisms by oscillating, rotating, and gradient magnetic fields, providing new solutions for overcoming the mucus barrier. In particular, the use of gradient magnetic fields is a simple and more accessible method for driving nanovehicles, without the need for complex fabrication techniques and expensive equipment.

Magnetically driven, muco-inert nanovehicles (Janus-MMSN-pCB) both enhanced the mucus penetration and mucosal epithelial cell endocytosis and can facilitate the development of mucosal carriers using a nanovehicle-based strategy. Furthermore, considering that both Janus-MMSN and Janus-MMSN-pCB have a Janus-type structure with a magnetic head and a non-magnetic body, they could move under not only gradient magnetic fields but also rotating magnetic fields, near-infrared radiation, and ultrasonic fields. These nanovehicles can move in any direction with a magnet under magnetic propulsion.

Though most mucus barriers in the human body have irregular forms and directions, it has been proven that the particles with enhanced motion speeds in mucus greatly increase the mucus penetration. Furthermore, the magnetic properties of the nanoparticles add an extra level of control in terms of the traveling speed and direction. For example, nanomagnetosols, in combination with a target-directed magnetic gradient field, achieved the targeted aerosol delivery to the lungs of mice. Therefore, in practice, the nanovehicles can be formed into aerosols or suppositories, treating the patient's mucosal sites with the guidance of an external magnetic field. The magnetic field can be provided by electromagnets or permanent magnets, positioned on the exterior surface of the patient's body and controlled by adjusting the strength and frequency of the magnetic field. This weakened improvement may be due to the assembly of the nanovehicles into micrometer-sized aggregates after the application a magnetic field for a long time, which cannot easily pass through the polycarbonate membrane with a pore size of 3.0 μm. The results indicated that the improvement yielded by the magnetic fields is evident for a short time, but it is not as large as that of the pCB coating for a long time.

This proved that zwitterionic polymer coatings can increase cellular uptake, as described in previous reports. In addition, the nanovehicles treated with magnetic fields showed a higher cellular uptake than the nanovehicles untreated with magnetic fields because of the magnetic-driven ability of Janus-MMSN and Janus-MMSN-pCB,. Notably, although both Janus-MMSN and Janus-MMSN-pCB could efficiently enter the Calu-3 cells under the drive of magnetic fields, Janus-MMSN-

pCB exhibited a much better cellular uptake. These results revealed that Janus-MMSN-pCB exhibited an excellent performance in its cellular uptake with a magnetic field treatment. In short, Janus-MMSN-pCB possesses a low cytotoxicity and enhanced cellular uptake.

The electrical neutrality and superhydrophilicity of the zwitterionic polymers (pCB) can effectively prevent Janus-MMSN-pCB from adhering to mucin, and the magnetic-driven ability can respond to an external magnetic field so as to cause the nanovehicles to move quickly through the mucus at a speed. The good biocompatibility and superior mucus penetration performance of Janus-MMSN-pCB prove that the combination of MPP and nanovehicles is an effective strategy for constructing efficient carriers for the purpose of transmucosal material delivery and provides a potential carrier for the diagnosis, treatment, and imaging of mucosal-related diseases.

Atomized respiratory administration (aerosol inhalation, for instance) is an efficient drug delivery alternative with lung as the main target organ. Compared with intravenous injection, inhalation has advantages such as convenient application, rapid onset, high local drug concentration, less dosage and low systemic exposure so as to reduce potential side effects. This will undoubtedly provide a new strategy for the treatment of COVID-19 pulmonary infection. Not surprisingly, therapeutic candidates with intrinsic short pharmacokinetics in the blood, such as small molecule inhibitors, fragmented antibodies, and nanobodies have been developed for aerosol inhalation administration to treat COVID-19. On the other hand, there are few studies on atomization inhalation administration of full-length antibodies with much longer $T_{1/2}$ in the blood, presumably due to developmental challenges and concerns of antibody aggregation or degradation after atomization. In fact, few clinical study has been conducted to date with inhaled antibodies

Therapeutic neutralizing antibodies administered by intravenous infusion or intramuscular injection don’t transport efficiently through the plasma-lung barrier to inhibit viral infection in lung. This implies that intravenous infusion is not an efficient method for antibody delivery to the lung for treating respiratory infectious diseases such as COVID-19. Inhalation of nebulized antibody aerosol into lung could potentially reach a much higher local antibody concentration at a relatively low dose, and hence could achieve better antiviral efficacy and reduce dose to increase affordability of costly antibody treatments and a single dose of nebulized antibody inhalation could be sufficient to treat most COVID-19 patients.

A potential risk with aerosol inhalation administration of neutralizing antibodies is the potential toxicity caused by massive accumulation of macromolecules in the lungs causing immunogenic reaction.

I. Biological Drugs

The lung can serve as a portal for the entry of biological drugs intended for the systemic circulation, which is exemplified by the development of inhaled insulin.

In the last decade, biological drugs have rapidly proliferated and have now become an important therapeutic modality. This is because of their high potency, high specificity and desirable safety profile. The majority of biological drugs are peptide- and protein-based therapeutics with poor oral bioavailability. They are normally administered by parenteral injection (with a very few exceptions). Pulmonary delivery is an attractive non-invasive alternative route of administration for local and systemic delivery of biologics with immense potential to treat various diseases, including diabetes, cystic fibrosis, respiratory viral infection and asthma, etc. The massive surface area and extensive vascularization in the lungs enable rapid absorption and fast onset of action. Despite the benefits of pulmonary delivery, development of inhalable biological drug is a challenging task. There are various anatomical, physiological and immunological barriers that affect the therapeutic efficacy of inhaled formulations.

Biological drugs (also known as biologics) are a diverse group of therapeutic agents which are generally large and complex molecules produced through biotechnology. Therapeutic peptides and proteins, including antibodies, constitute the largest group of biological drugs and are the focus of this review. Over 25% of novel drugs approved by the FDA between 2015–2019 were biologics covering a broad range of indications, including genetic disorders, auto-immune diseases, cancers, asthma and allergic diseases. The clinical and commercial success of biological drugs is attributed to their high target binding affinity, high specificity of action and desirable safety profile. However, due to their large molecular size and high polarity, the permeability of biologics through the intestinal epithelium is low or even negligible. In addition, enzymatic degradation by peptidases and proteinases in the gastrointestinal tract renders them orally inactive. Biological drugs are therefore currently largely administered by injections. Injectable drug therapy however is painful and inconvenient for patients, especially when drugs are used for chronic conditions. At least 10% of patients in the world suffer from needle phobia leading to poor compliance.

Despite these potential advantages, the development of inhalable biological drugs is challenging. Inhalable formulations need to be rationally designed to achieve appropriate aerodynamic properties for effective lung deposition. The geometry of

the airways, humidity, mucociliary clearance and alveolar macrophages are essential in maintaining the sterility of the lung and subsequently pose critical barriers to the therapeutic efficiency of inhaled formulations. In addition, inhaled biologics should exhibit favorable biophysical properties to withstand the stresses encountered during production, aerosolization and transportation. Moreover, if systemic therapy is desired, inhaled biologics must be able to cross the lung epithelium and reach the blood circulation in sufficient levels in order to exert a therapeutic effect.

There are several anatomical, physiological and immunological barriers that affect the delivery efficacy of inhaled biologics, including the highly branched structure of the airways, mucociliary clearance, macrophages uptake, pulmonary surfactant, alveolar epithelium permeation and enzymatic metabolism.

Anatomical Barriers

Being able to deliver a sufficient dose of biological drug to the human lung is challenging. The highly branching structure of the lung poses the primary barrier for inhaled particles deposition. The efficiency and the site of deposition of aerosol particles in the lung is significantly affected by their physicochemical properties, including the aerodynamic particle size, shape, charge, hygroscopicity and density. The aerodynamic diameter is the key parameter that determines the lung deposition efficiency. Again, it is defined as the diameter of a sphere with a unit density that has the same terminal settling velocity in still air as the particle in consideration. In general, particles with the aerodynamic diameter between 1 and 5 μm are deposited in the lower respiratory tract, whereas those with size over than 10 μm are deposited in the oropharyngeal region. Particles smaller than 1 μm are exhaled during tidal breathing.

The optimal site in the lung for the deposition of inhaled biologics is not fully clarified. Aerosol particles must dissolve to release the active drug for subsequent pharmacological action and absorption. However, the amount of fluid in the lung for particle dissolution is limited. The estimated volume of lung fluid is 10 to 30 mL in humans. It is difficult to predict the volume of fluid that an inhaled aerosol particle is exposed to after deposition. In addition, the thicknesses of the lung lining fluid layer is different between central and peripheral lungs. As the airways gradually become smaller in diameter, the lung lining layer becomes thinner until it reaches the alveoli. Particles deposited in the upper airways tend to dissolve quicker because of the larger solid-liquid interface than in alveolar space

The peripheral airways have a significantly larger absorptive surface than the conductive airways and is considered to be the target region of systemically acting

biologics. Epithelial permeability in the upper airway is less favorable because of smaller surface area and reduced regional blood supply.

Mucociliary clearance is a dominant defense mechanism in the upper conduction airways against potentially harmful particles. It exerts its function by integrative activity of non-cellular (the mucus layer) and cellular elements (the cilia and secretory cells). In healthy subjects, inhaled insoluble particles deposited in the airways are swept up and cleared from the respiratory system within 24 hours. Furthermore, since peptides and proteins are highly charged and hydrophilic, mucus components tend to interact with them and retard their diffusion, thereby limiting drug absorption. For instance, there is an electrostatic repulsion between the negatively charged protein molecules and mucin fibers, subsequently preventing them from a close contact with the lung epithelium.

In the peripheral lungs, macrophage uptake plays a significant role in alveolar clearance of inhaled biological drugs. After being phagocytosed, inhaled particles could be either degraded by intracellular enzymatic lysosomal system; transported to the lymphatic system; or migrated along the ciliated airways and removed from the respiratory tract by mucociliary clearance. The uptake by alveolar macrophages is size-dependent. Particles with geometric diameter range between 1–2 μm are easily phagocytosed by macrophages (15–22 μm in diameter) and those with smaller size are taken up less effectively

Pulmonary Surfactant

Pulmonary surfactant (PS) is a continuous liquid layer spreading from the distal to the proximal lungs. It is the first contact for aerosol particles deposited in the airways and the fate of particles depend on their interaction with the PS layer. The composition of PS includes phospholipids (~92%, by mass) and surfactant proteins (~8%). Large proteins may interact with PS components, triggering aggregation and subsequent macrophage degradation. PS was conventionally considered as a barrier for drug delivery in the peripheral lung, where mucus is absent in healthy condition. On the other hand, PS has been investigated as a carrier to promote the transportation of drugs in the airways. It was shown that natural PS and its most abundant phospholipids, dipalmitoylphosphatidylcholine (DPPC), are potential absorption enhancer of inhaled peptides and proteins

The absorption enhancing effect of PS probably was mediated by opening the tight junction of the cell monolayer to accelerate the paracellular transport of hydrophilic protein molecules. Little is known about the interactions between surfactant biomolecules and inhaled biologics at a molecular level and this gap in knowledge necessitates further research.

Airway Epithelium

Inhalation has been investigated for local or systemic delivery of biological drugs. For locally acting drugs, inhaled formulation needs to solubilize in the pulmonary lung fluid to interact with the mucus and target cells in the airways in order to elicit the pharmacological effect. For systemically acting biological drugs, the drug molecules must be able to gain access to the systemic circulation in addition to proper lung deposition and dissolution. The pulmonary epithelium is the primary barrier for the transportation of protein drugs to the bloodstream. Transportation of biological drugs across respiratory epithelium is size-dependent. Small soluble peptides and proteins with molecular weight below 40 kDa are rapidly detected in the bloodstream, whereas those with molecular weight above 40 kDa are slowly absorbed in the lung over hours to days. Small peptides are rapidly absorbed, but in parallel are subject to substantial metabolism in the airways. Larger proteins, e.g., antibody fragments and mAbs, are absorbed very slowly with limited bioavailability.

There are two possible mechanisms for biologics to cross the pulmonary epithelium, namely paracellular transport and transcytosis. Peptides and proteins without specific receptors in the lung epithelial cells are transported by paracellular pathway or non-specific pinocytosis. A number of biological drugs are actively permeating the alveolar epithelium by receptor-medicated transcytosis.

As mentioned above, two major cell types are found in the alveolar epithelium, namely type I and type II pneumocytes, joined by tight junctions. Over 90% of the alveolar surface is covered by type I cells, while type II cells make up to 5–10% of the surface. Subcellular morphology study revealed that endocytic vesicles are present in type I cells, which are likely the major cell types for drug absorption in the lung. The relative contribution of type I and type II pneumocytes in overall transportation of peptides and proteins remain to be determined. It was reported that albumin (66 kDa) is predominantly taken up by receptor-mediated endocytosis in alveolar type II cells. Although type II cells occupy only a small proportion of surface area in alveoli, the uptake of albumin in type II cells is higher than type I cells. Given the role of endocytosis/transcytosis in transepithelial trafficking of inhaled biologics, identification of specific receptors involved in the cellular uptake and understanding the transport mechanism are important for the development of efficient pulmonary delivery strategies for biological drugs.

Metabolism

Many types of protease and peptidase are found in the airspace and epithelial cells. Alveolar macrophages and other inflammatory cells (e.g., neutrophils) are the sources of proteases. The extent to which protein molecules are metabolized is unclear. The level of proteases is higher in the airways in inflamed lung; thus, the

efficacy of aerosolized proteins and peptides may be impaired. Enzymatic hydrolysis of small natural peptides (less than 3 kDa) is high unless they are chemically modified to block the activity of peptidase. As the molecular weight increases, proteins with greater tertiary and quaternary structure could inhibit peptidase hydrolysis. In general, proteins with molecular weights between 6 and 50 kDa are relatively resistant to most peptidases and have good bioavailability upon inhalation. Formulations that retard enzymatic degradation or promote drug absorption can increase bioavailability. Addition of protease inhibitors to the formulation was shown to improve the pulmonary absorption and bioavailability of peptides and proteins. Given the potential toxicity, safety is a major concern of formulations with protease inhibitors, especially in chronic use.

The majority of the biological drugs on the market have peptidic backbone ranging from small peptides to monoclonal antibodies (mAbs). The major therapeutic mAbs are immunoglobulin G (IgG), which have a long serum half-life of approximately 10–21 days in humans resulting from the physiological recycling mechanism mediated by the neonatal Fc receptor (FcRn). Topical delivery of full-length mAbs via inhalation has been investigated in animal models and the therapeutic efficacy in respiratory diseases, including lung cancer, asthma and pulmonary intoxication, are demonstrated. In addition, pulmonary delivery of antibody fragments, such as antigen-binding fragment (Fab), domain antibody (dAb) and single-domain antibody (Nanobody®), have also been explored.

Biologics are highly labile and prone to degradation when exposed to various types of external stresses, such as elevated temperature, extreme pH, freezing stresses, organic solvent, salt, shear force and light exposure, etc. Aggregation is the most common and troublesome manifestation of protein instability. It has been observed at all stages of protein product development, leading to reduced bioactivity or increased risk of immunogenicity. Fibrillation is a specific protein aggregation state that can occur naturally in human etiology. Several neurodegenerative diseases, such as Alzheimer's disease and Huntington's disease, are characterized by the formation of protein fibrils (also known as amyloid proteins), an unbranched protein fiber with repeating cross-β sheet structure. Inhaled insulin has been reported to aggregate rapidly and form toxic pulmonary amyloid aggregates at the air-tissue interface. The formation of amyloid deposits causes pulmonary dysfunction after insulin inhalation

This finding suggests that the large surface area and the air-tissue interface in the lungs may promote conformational rearrangement of proteins with misfolding tendency. Therefore, caution should be paid when a protein with amyloidogenic nature is designed to be delivered to the lungs via inhalation.

Proteinaceous drugs with exogenous sequences can be recognized as non-self by the host immune system. Immunogenicity is characterized by the development of anti-drug antibodies (ADAs). The formation of immune complexes between ADAs and the protein therapeutics may accelerate drug clearance, neutralize therapeutic efficacy and induce hypersensitivity reactions. This finding led to the hypothesis that inhaled mAbs may be more immunogenic than those administered parenterally.

From the delivery point of view, most peptide and protein-based drugs display their pharmacological effect by interacting with cell surface receptors or extracellular ligands, thus the objective of biological drug delivery is to maintain the drug concentration at extracellular sites within the therapeutic window for sufficient period of time. Biologics are also associated with a high price tag (from thousands to hundreds of thousands of US dollars per patient per year). Effective biologics delivery systems can potentially lower the administration cost, thus enhancing patient's and healthcare system's affordability. The unique structure and characteristics of biologics separate them as a special group of therapeutics.

Strategies for Inhaled Delivery of Biological Drugs

Various approaches have been applied to modify the structure of biological drugs to enhance pulmonary absorption or maintain in vivo stability, such as antibody fragment development, Fc engineering and PEGylation. Antibody fragments offer the advantages of enhanced tissue penetration, easy and inexpensive production. Given the lack of an Fc region, antibody fragments are subject to rapid degradation with short serum half-life in vivo. Several antibody fragments are developed and investigated for pulmonary delivery. The pharmacologically active antigen-binding region is preserved in the Fab, which has been delivered to the lungs via inhalation to neutralize inflammatory cytokines.

Domain antibodies (dAbs) are antibody fragments derived from the variable domains of either the heavy or light chain of human IgG. These are the smallest functional unit of human antibodies with antigen-binding activity. The investigation of pulmonary delivery of dAbs was focused on the prophylaxis and treatment of acute lung injury (ALI). Specific inhibition the signaling of tumor necrosis factor (TNF) receptor-1 (TNFR1) via inhaled dAbs significantly reduced the airway inflammation in animal (mice and cynomolgus monkeys) and human models of ALI.

Nanobodies (a registered trademark of Ablynx, Ghent, Belgium) are recombinant single-domain antibodies derived from the heavy chain-only antibodies (HCAbs) of Camelidae. Nanobodies combines the advantages of small-molecule drugs such as smaller size, good stability and ease of production, with the characteristics of high selectivity and affinity of conventional antibodies. These biophysical

characteristics make Nanobodies especially relevant to antiviral therapeutics, e.g., RSV infection, influenza virus infection and Middle East respiratory syndrome coronavirus infection (MERS-CoV) and COVID-19. ALX-0171 is a 42-kDa trimeric Nanobody designed to target the RSV surface fusion (F) protein. It inhibits the release of RSV from the apical surface in cell culture

Recently, the COVID-19 pandemic encourages the exploration of novel treatment strategies against viral infection. The cellular entry of SARS-CoV-2 is achieved by binding of viral spike glycoprotein to the angiotensin converting enzyme 2 (ACE2) receptor on the surface of host cell. Nanobodies, e.g., Nb11-59 (15 kDa, Novamab Biopharmaceuticals, Shanghai, China) and Ty1 (12.8 kDa, Karolinska Institutet, Stockholm, Sweden), are developed to target the receptor binding domain (RBD) of SARS-CoV-2 spike glycoprotein, blocking the virus entry. These nanobodies exhibited potent viral neutralizing activity in vitro and remained stable during nebulization. Inhaled nanobodies are promising antiviral therapy against COVID-19 viral infection.

PEGylation

Enzymatic degradation and rapid lung clearance are two significant challenges faced by inhaled biologic therapies in the treatment of respiratory diseases. Prolonging the retention of inhaled biomolecules could be beneficial due to an altered pharmacokinetic profile (longer duration of local effects) and reduced dosing frequency. Conjugation of one or more hydrophilic polyethylene glycol (PEG) chains to peptides and proteins can increase the molecular mass and provide shielding effect to the conjugated molecules. PEGylation protects proteins from renal clearance and proteolytic enzyme degradation, subsequently prolongs the protein residency in the body. PEGylation has been demonstrated to be an effective method to extend the retention time of therapeutic proteins in the lung, including human alpha1 proteinase inhibitor (α_1-PI, also known as alpha-1-antitrypsin, AAT), IFNα and antibody fragments. The PEGylated Fab fragment, anti-IL-17A PEG40-F(ab′)$_2$, has display improved efficacy in reducing biomarkers and lung inflammation in a murine model of allergic asthma. PEGylation enhances the stability of antibody fragments in the airways by mucoadhesion, avoidance of alveolar macrophage uptake and decreased transepithelial transport to the bloodstream.

The residence time of PEGylated antibody fragments was highly dependent on the deposition site in the respiratory tract; the deeper the deposition, the longer the residency. The prolonged residency of proteins in the deep lungs is attributed to the decreased mucociliary clearance activity from the central to distal airways. The number and size of the PEG chains, as well as the site of PEGylation are key parameters in determining the bioactivity and pharmacokinetic properties of

conjugated proteins. Optimization of PEGylation is required in each delivery system to confine exposure of the biologics to the lungs so as to minimize systemic adverse effects and repeated administration.

PEGylation is a chemical process involving the attachment of polyethylene glycol (PEG) chains to a molecule of interest through covalent binding or non-covalent complexation; Fc-fusion proteins are composed of the Fc domain of IgG genetically linked to a peptide or protein of interest. The first-generation Fc-fusion proteins are dimeric wherein an effector molecule is fused with each arm of the Fc fragment. The second-generation Fc-fusion proteins are monomeric wherein a single effector molecule is conjugated to one arm of the Fc fragment.

Fc Engineering

Proteinaceous molecules can be linked to the Fc fragment of IgG to produce Fc-conjugated or Fc-fusion proteins. These hybrid biomacromolecules have prolonged plasma half-life because of FcRn-mediated recycling in the blood vessel endothelium

Inhalation Technology

Successful delivery of inhaled biological drugs requires not only a proper formulation but also an inhaler device to generate drug aerosols. Due to the complex structure of biological drugs, the aerosolization process is required to produce aerosol particles with aerodynamic properties suitable for inhalation, and at the same time preserve the physical integrity and potency of drug molecules. Moreover, patient compliance and acceptance are critical as part of disease management in inhalation therapy.

Nebulization has drawbacks such as low delivery efficiency, long administration time, poor reproducibility and relatively high costs of device maintenance. Nevertheless, it also provides several considerable advantages, including the avoidance of drying process during manufacture and suitability for patients of different ages and stages of illness. Ultrasonic nebulizers, jet nebulizers and vibrating-mesh nebulizers are the three major types of nebulizers used in the management of lung diseases. Protein aggregation and unfolding at the air-liquid interface are especially challenging during aerosolization process in ultrasound and jet nebulizers, while vibrating mesh nebulizers are more suitable for biological drugs as they do not produce heat and are less likely to denature the molecules. The drug and the device should be optimized together to achieve desirable aerosol performance and retain the molecular integrity of biologics. Nebulization of Nanobody® ALX-0171 via jet nebulizer triggered significant protein multimerization and aggregation, which were barely detected in vibrating mesh nebulizer.

Smart nebulizers that provide accurate dosing independent of lung function are in development. They are electronically regulated inhalation systems coupling an electronic control unit with a vibrating mesh nebulizer. These devices control the inhalation by the patient's breathing patterns and generate inhalation parameters unique to the individual. These devices control the inhalation by the patient's breathing patterns and generate inhalation parameters unique to the individual.

AKITA^2 APIXNEB inhalation system delivered human α_1-PI (Prolastin-C, Grifols, Barcelona, Spain) to the lungs of CF patients and achieved a high overall lung deposition (around 70% of the drug loaded into the nebulizer), irrespective of the severity of lung function impairment.

PMDIs use propellants to generate aerosol for inhalation. Drugs are either dissolved or suspended in a single propellant or propellant mixture together with excipients such as co-solvents and surfactants. To date, there is no approved pMDI product for inhaled biologics therapy. Nevertheless, biologics formulations for pMDIs are being investigated. Proteins and peptides are generally hydrophilic and therefore are poorly soluble in non-polar hydrofluoroalkane (HFA) propellants. Biologics are formulated as suspension rather than solution in pMDI formulation, which substantially limits the formulation possibility. Poor solubility of biologics in the propellants also limits the dose range that can be delivered per actuation

A major concern is the denaturation of proteins and peptides when they interact with the propellants. To enhance the stability, therapeutic proteins could be incorporated in a particulate carrier to suspend in a propellant

Dry Powder Inhalers (DPIs)

DPIs are propellant-free, portable and easy-to-use inhalation device. When considering DPIs for biologics, it is important to identify a suitable drying method that can produce particles with good aerosol property and preserve the integrity of the biological drugs. While milling is the most commonly used technique to generate inhalable dry powders, this approach is not suitable for fragile molecules that are prone to degradation

Spray drying (SD)

Spray drying is a single-step, continuous and scalable particle processing technique in the pharmaceutical industry. It has been employed to produce inhalable dried powders of biologics, such as insulin, DNase, anti-IgE mAb, hGH, IgG1 and infliximab. During SD, liquid feed is atomized into a hot drying gas where the solvent is evaporated to produce dried particles. Thermal stress and high shear force may denature and hence inactivate biological molecules.

Spray freeze drying (SFD)

Is another drying technique that combines SD and freeze drying. A liquid is first atomized into a cryogen (usually liquid nitrogen), followed by lyophilization. This method is less well-established in pharmaceutical industry compared to SD due to scale-up difficulties. However, the SFD particles usually exhibit porous structure with good aerosol performance, making them particularly attractive for inhalation. The avoidance of high temperature also makes SFD suitable to generate inhalable dry powders of thermolabile materials like vaccines with retained potency and immunogenicity.

J. Nanobodies

Beyond mAb fragments, novel protein scaffolds such as Nanobodies are causing quite a stir of excitement in the field of protein-based therapeutics. Due to the smaller size and simpler structure of protein scaffolds, they have easier access to binding sites on enzymes, greater tissue penetration and accumulation, and enhanced thermostability in comparison to full-length mAbs.

In 2018, Colasuonno et al. proposed an “armed” version of DPN against ischemic stroke, a dangerous medical condition wherein the cerebral blood supply is impeded. The reduction in vascular flow due to the formation of a clot or an embolus causes a damage to the surrounding brain tissue, in that cerebral tissue, due to its high metabolic and energy needs to function properly, is highly sensitive to the lack of oxygen and glucose. The duration of the vascular occlusion, together with the location of the occlusion in the vascular network (main arterial occlusion or in microcirculation), is directly related to the severity of the damage. In the cerebral tissue, hypoxia and the lack of glucose block the fine regulation of ions and neurotransmitter trafficking in neurons. Cell depolarization and accumulation of water, ions, and neurotransmitters in the extracellular space increase brain edema, inflammation, cell excitotoxicity, and apoptosis. Thus, stroke is a difficult-to-treat condition, where the timing of treatment is crucial to reduce the negative effects of the event.

Ischemic Stroke

Endothelial cells, during cerebral ischemia, release plasminogen activators in the intravascular space to promote plasmin-induced lysis of clots. Plasminogen activators are also released at the same time by perivascular astrocytes located in the cell-basement membrane-astrocyte interface which affects the endothelium and increases the permeability of the blood–brain barrier (BBB). In the armed-DPN approach, DPNs are conjugated with tissue plasminogen activator (tPA), the most commonly used treatment for patients with acute ischemic stroke. tPA is a serine protease that induces the conversion of plasminogen to plasmin, triggering the lysis

of fibrin clots. Although tPA is a life-saving drug, it has been shown since the first clinical administration that the use of this treatment is beneficial within a short time interval of up to 4.5 h after the ischemic event. Conversely, delayed administration of tPA increases the occurrence of cerebral hemorrhage and other negative side effects. It has been found that following ischemia and increased permeability of the BBB, tPA can extravasate and accumulate in the brain tissue, where it can act as a cell stimulus, worsening the condition of excitotoxicity and increasing cell death. tPA-DPNs therefore have multiple advantages: the conjugation of the drug on the surface of the particles prevents the undesired spilling of the drug from the blood vessels (even under condition of increased vascular permeability), the conjugation of tPA on particles is stable (less of the 10% of the drug can detach from the particle), and lastly conjugation tPA to DPNs protects the drug from inactivation by serum proteins, thereby preserving its pharmacological activity.

It has been observed that 70% of the original activity of tPA is maintained even after 3 h of incubation of tPA-DPN in FBS. The efficacy of tPA-DPN has been confirmed in vitro by thrombolysis tests under both static and dynamic experimental conditions and in vivo in the murine model of mesenteric vein thrombosis. Under static in vitro experimental conditions, the dissolution of the clots is comparable with free tPA at the same concentration. However, under dynamic conditions using a double-channel microfluidic chip to simulate clot formation and dissolution, tPA-DPN reduced the clot area by about 50% after 60 min, faster than free-tPA which took 90 min to achieve the same effect. This last result was also confirmed in the mesenteric mouse model, in which the 2.5 mg/kg concentration of tPA-DPN showed more efficacy than free-tPA to recanalize clotted vessels. In fact, 2.5 mg/kg of tPA-DPN recanalize 90% of the occluded vessels with a 50% reduction of the clot size in 35 min. Conversely, free-tPA at the same concentration recanalize 40% of the vessels, with a 20% reduction of the clot size in 35 min. All these experimental evidence can be attributed to a combination of features of the DPN as size, shape, deformability, and adhesive interactions with fibrin of the blood clot. The combination of size and shape leads to the margination of the particles near the vessel walls, favoring the interaction with the endothelium and the clot.

The Young's modulus of these particles is similar to that of cells and ranges between few to a few hundreds of kPa. The softness of DPN can promote the trapping and accumulation of these particles inside the fibrin network. Moreover, the presence of tPA on the surface increases the adhesiveness of the particles to the clot due to the significant affinity between tPA and fibrin, and for the possibility of having multivalent interactions between the various fibrin molecules in the clot and the multiple tPA molecules on the particle surface. Furthermore, in the presence of

more permeable BBB, the stable conjugation of tPA on DPN prevents the drug from exiting the damaged and more permeable vessels. These features, in addition to the experimental data obtained, are a good premise and show how this DPN technology is a good basis for future applications in vascular disease.

Cancer

Since Matsumura and Maeda reported on the enhanced permeability and retention (EPR) effect for the delivery of macromolecules in cancer therapy, there has been a prevailing philosophy to design nanomedicines for cancer imaging and treatment characterized by nanoparticles with a spherical shape, an average diameter of 100 nm, and a surface mostly decorated with polyethylene glycol (PEG) chains. Particularly, Maeda et al. observed in pre-clinical in vivo models that endothelial cells of tumor vessels are not tightly connected but they rather exhibit irregular fenestrations ranging in size from several tens up to a few hundreds of nanometers. This peculiar characteristic has stimulated scientists in developing a plethora of blood-borne spherical nanoparticles sufficiently small to pass through these fenestrations and be retained within the diseased tissue. This variety of nanoparticles relies on self-assembly and colloidal interactions and differ in their material compositions, sizes, and surface properties. Specifically, both organic (e.g., lipids, polymers, block copolymers), and non-organic (e.g., iron oxide, gold, silver) materials have been employed. Nanoparticle surfaces have been modified with different coatings, including lipids, stealth polymer chains that enhance particle blood longevity, and a variety of moieties for recognizing specific cancer cell molecules enabling what is known as active targeting.

Indeed, the US food and Drug Administration approved the first EPR-dependent nano-drug, known as Doxil, in 1995, putting in the spotlight nanotechnology and its benefits in the fight against cancer. However, the universal utility of the EPR effect in the fight against cancer has recently been re-scrutinized, and alternative delivery strategies are necessary to facilitate the delivery of therapeutics to tumors.

Particularly, recent studies have shown that the EPR effects might not be as clinically relevant as it is in mice, as the size of the irregular vascular fenestrations and their density depend on the type and the stage of the tumor. These data have stimulated scientists to explore vascular targeting as a complementary therapeutic option. This more general vascular targeting delivery strategy is supported by another hallmark of tumor physiology, regardless of cancer type and stage: the disorganized vascular architecture leading to impairment of blood perfusion.

In this field, it has been extensively demonstrated the need to finely tune nanoparticle size, shape, surface properties, and mechanical stiffness, the so-called

4S parameters, to boost tumor accumulation. Following first in silico and in vitro studies with in vivo tumor models, the discoidal shape and a micrometric size appears as the optimal parameters for nanoparticles to enhance their deposition in tumor vasculature by mitigating hemodynamic forces which can dislodge the particles.

Once in circulation, discoidal nano-constructs, exhibit the propensity to drift laterally in the "cell-free layer," while spherical particles flow within the vessel core together with RBCs. Additionally, as compared to spheroids, discoidal particles present a larger surface suitable for interacting with the vessel walls. Regarding size, Decuzzi and collaborators demonstrated that discoids with nanometer size and thus a limited surface interaction would minimally feel the shear stress, thus accumulating in nonspecific areas. On the contrary, the interaction between the vessel walls and large discoids would be prevented by the same dislodging forces. Among the others, the mechanical stiffness has been revealed by the authors as the most critical parameter to control. Its role in controlling blood longevity, organ biodistribution, and tumor accumulation has been investigated.

These data would suggest that the deformability associated with soft DPNs is the main reason for the enhanced tumor accumulation, given the ability to escape from macrophage recognition and phagocytosis. Such a phenomenon should then prolong their circulation in the bloodstream, thus boosting the chances to accumulate into the disease area. Overall, it has been shown that the rational design of non-spherical nano constructs, by finely tuning the geometry and the mechanical properties, is a fundamental step for optimizing the nano construct performances in vivo, as compared to conventional bloodborne nanoparticles.

K. Activation of drug delivery systems

In general, the release of the drugs takes place in two distinct phases–an initial burst release followed by a prolonged and sustained release. When drugs are loaded in their molecular form, the release is governed more by the intrinsic properties of the drug. It was possible to study the release kinetics of DEX from μPLs using two different kinds of loading. When the drug was loaded in its molecular form, 60% of the drug was released in the first 24 h while the remaining drug was released slowly over 10 days. However, when employing the hierarchical system (i.e., loading DEX into smaller PLGA particles which are subsequently loaded into μPLs), the release was further slowed and only 35% of the drug was released in the first 8 h. Another in-depth investigation was carried out to understand the effect of polymer concentration, shape, and surface area on the loading and release kinetics of curcumin and DEX. Greater amounts of PLGA

resulted in more accurately shaped particles, while less PLGA resulted in more defect-prone particles. The amount of drug loaded into the particles was found to be directly proportional to the amount of the polymer present in the particles, i.e., taller and denser particles were loaded with higher amount of curcumin. The loading efficiency of DEX and curcumin also was directly proportional to the hydrophobicity of the drug molecules. Notably, higher polymer content corresponded to a slower release rate and reduced the initial burst release. This observation can be attributed to the more compact polymeric matrix of the particle.

In summary, a systematic study of μPLs loading and release of multiple therapeutics highlights the potential of this delivery platform with controlled geometry. The particle size and shape can be further explored as a means for imparting a therapeutic benefit (beyond the drug delivery aspect) in the treatment of diseases.

II. HYPERINFLAMMATORY CONDITIONS

Vertu Realities will focus on novel corporeal countermeasures to reduce the consequences of excessive Inflammation on internal and external vital tissues. As previously stated, we must therefore target the effector cells that drive systemic inflammation, causing direct tissue damage and secreting a range of pro-inflammatory cytokines that initiate and propagate imbalanced immune responses.

Methods referred to herewithin can be used for treating and/or preventing a number of conditions that are associated with inflammation. As used herein, the term “inflammatory condition,” includes any inflammatory disease, any inflammatory disorder, and/or any leukocyte activated disorder wherein the organism's immune cells are activated. Such a condition can be characterized by a persistent inflammatory response with pathologic sequelae and/or by infiltration of leukocytes, for example, mononuclear cells and neutrophils, leading to tissue destruction.

Inflammatory conditions

Include primary inflammatory diseases arising within a subject and/or secondary inflammatory disorders arising as a response to a medical procedure. The Bio-Atmosphere systems, devices, and methods discussed can treat any inflammatory condition for any subject. As used herein, the term “subject” refers to any animal (e.g., a mammal), including, but not limited to, a human (e.g., a patient), non-human mammals, for example, a non-human primates and other experimental

animals, farm animals, companion animals, and the like, which is to be the recipient of a particular diagnostic test or treatment.

Accordingly, the petitioned BioZone Bio-Atmosphere discussed herein may treat and/or prevent any inflammatory condition, including primary inflammatory diseases arising within a subject and/or secondary inflammatory disorders arising as a response to a medical procedure (e.g., dialysis or cardio-pulmonary bypass). Examples of applicable inflammatory conditions, including inflammatory diseases and/or disorders, include, but are not limited to, systemic inflammatory response syndrome (SIRS), polyarteritis, Wegener's granulomatosis, autoimmune vasculitis, anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, extracorporeal membrane oxygenation (ECMO), cardiopulmonary bypass syndrome, acute respiratory distress syndrome (ARDS), acute lung injury (ALI), chronic obstructive pulmonary disease (COPD), sepsis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, multiple sclerosis (MS), psoriasis, allograft rejection, asthma, acute renal failure, chronic renal failure (CRF), end stage renal disease (ESRD), cardiorenal syndrome (CRS), chronic heart failure (CHF), stroke, myocardial infarction (MI), hepatorenal syndrome, cirrhosis of the liver, diabetes mellitus (type 2 diabetes), and acute organ failure from ischemic reperfusion injury to myocardium, central nervous system, liver, kidney, or pancreas.

Additional examples of inflammatory conditions include, but are not limited to, transplant (such as organ transplant, acute transplant, xenotransplant) or heterograft or homograft (such as is employed in burn treatment) rejection; ischemic or reperfusion injury such as ischemic or reperfusion injury incurred during harvest or organ transplantation, myocardial infarction or stroke; transplantation tolerance induction; arthritis (such as rheumatoid arthritis, psoriatic arthritis or osteoarthritis); respiratory and pulmonary diseases including but not limited to chronic obstructive pulmonary disease (COPD), emphysema, and bronchitis; ulcerative colitis and Crohn's disease; graft vs. host disease; T-cell mediated hypersensitivity diseases, including contact hypersensitivity, delayed-type hypersensitivity, and gluten-sensitive enteropathy (Celiac disease); contact dermatitis (including that due to poison ivy); Hashimoto's thyroiditis; Sjogren's syndrome; Autoimmune Hyperthyroidism, such as Graves' Disease; Addison's disease (autoimmune disease of the adrenal glands); Autoimmune polyglandular disease (also known as autoimmune polyglandular syndrome); autoimmune alopecia; pernicious anemia; vitiligo; autoimmune hypopituitarism; Guillain-Barre syndrome; other autoimmune diseases; glomerulonephritis; serum sickness; urticaria; allergic diseases such as respiratory allergies (hay fever, allergic rhinitis) or skin allergies; scleroderma; mycosis fungoides; acute inflammatory and respiratory responses (such as acute respiratory distress syndrome and ischemia/reperfusion injury); dermatomyositis; alopecia areata; chronic actinic

dermatitis; eczema; Behcet's disease; Pustulosis palmoplanteris; Pyoderma gangrenosum; Sezary's syndrome; atopic dermatitis; systemic sclerosis; morphea; trauma, such as trauma from a gun, knife, automobile accident, fall, or combat; and cell therapy, such as autologous, allogenic or xenogeneic cell replacement. The addition of Radiation Induced Lung Injuries and the manifestations thereof is now added to this itemization.

Leukocytes,

Especially neutrophils, are major contributors to the pathogenesis and progression of many clinical inflammatory disorders, including systemic inflammatory response syndrome (SIRS), sepsis, ischemia/reperfusion injury, acute respiratory distress syndrome (ARDS) and acute kidney injury (AKI). Cardiac surgical advances have been dependent upon the techniques for cardiopulmonary bypass (CPB). It has been recognized that a systemic inflammatory response occurs in association with CPB, resulting in multiple organ dysfunctions (MOD) following surgery. Multiple insults during CPB have been shown to initiate and extend this inflammatory response, including artificial membrane activation of blood components (membrane oxygenator), surgical trauma, ischemia-reperfusion injury to organs, changes in body temperature, blood activation with cardiotomy suction, and release of endotoxin. These insults promote a complex inflammatory response, which includes leukocyte activation, release of cytokines, complement activation, and free-radical generation. This complex inflammatory process often contributes to the development of acute lung injury, acute kidney injury, bleeding disorders, altered liver function, neurologic dysfunction, and ultimately MOD. Leukocytes, for example, neutrophils, are major contributors to the pathogenesis and progression of many clinical inflammatory conditions, including systemic inflammatory response syndrome (SIRS), sepsis, ischemia/reperfusion injury and acute respiratory distress syndrome (ARDS).

Several different and diverse types of leukocytes exist; however, they are all produced and derived from a pluripotent cell in the bone marrow known as a hematopoietic stem cell. Leukocytes, also referred to as white blood cells, are found throughout the body, including in the blood and lymphatic system. There are several different types of leukocytes including granulocytes and agranulocytes. Granulocytes are leukocytes characterized by the presence of differently staining granules in their cytoplasm when viewed under light microscopy. These granules contain membrane-bound enzymes, which primarily act in the digestion of endocytosed particles. There are three types of granulocytes: neutrophils,

basophils, and eosinophils, which are named according to their staining properties. Agranulocytes are leukocytes characterized by the absence of granules in their cytoplasm and include lymphocytes, monocytes, and macrophages.

Platelets

Or thrombocytes, also contribute to inflammatory conditions, as well as to homeostasis. Upon activation, platelets aggregate to form platelet plugs, and they secrete cytokines and chemokines to attract and activate leukocytes. Platelets are found throughout the body's circulation and are derived from megakaryocytes.

The molecules that are primarily responsible for initiation of leukocyte and platelet adhesion to endothelium are P-selectin and von Willebrand factor, respectively. These molecules are found in the same granules, known as Weibel-Palade bodies, in endothelial cells. Upon activation of endothelial cells, the Weibel-Palade bodies migrate to the cell membrane to expose P-selectin and soluble von Willebrand factor at the endothelial cell surface. This, in turn, induces a cascade of leukocyte and platelet activity and aggregation.

III. CYTOKINE STORM

Cytokines are small, cell-signaling proteins that are an integral component in the immune system’s armamentarium, regulating immune and inflammatory responses at the site of injury or infection. In many critical-care patients, a dysregulated cytokine response can cause hyperinflammation, known as a cytokine storm, and this can lead to tragic adverse outcomes unless mitigated in time.

To discuss the genesis of the cytokine storm we must recognize that inflammatory cytokines respond to injury or infection by increasing local blood flow and temperature and mobilizing immune cells to the site. Neutrophils and monocytes, which are leukocytes (white blood cells) that are integral to the inflammatory process, produce cytokines and are also recruited to an injured or infected site.

In a normal immune response, neutrophils are the first immune cells to arrive at the site and are key to the entire immune response through a variety of functions, including the production of neutrophil extracellular traps (NETs) that kill pathogens. Because neutrophils and monocytes have heterogenic properties, they are also integral to tissue remodeling and repair.

In a dysregulated immune response, overactive monocytes and NETs lead to the overproduction of cytokines, and neutrophil apoptosis may be delayed. Additionally, feedback mechanisms that regulate the immune system are altered, so negative feedback is all but absent while positive feedback is left unchecked, with cytokines continually recruiting immune cells. This results in damaging hyperinflammation at the site, but this is not where it stops.

To assess the destructive path of the cytokine storm, we recognize that local hyperinflammation spreads uncontrollably through the systemic circulation to other parts of the body, leading to endothelial dysfunction and consequent organ damage. Additionally, through organ crosstalk, damaged organs can cause further destruction to other organs yet even still these are not all the possible morbidities associated with the cytokine storm. Without effective treatment, patients can suffer from catastrophic organ failure and lose their lives.

The impact of Cytokines on the lungs and kidneys during the COVID-19 pandemic increased our understanding of the connection between the lungs and the kidneys. Loss of normal function in either organ can induce direct and indirect dysregulation of the other, as we have witnesses with COVID-19 patients.

To explain the damage interplay between organs with the COVID example we recognized there were two phases of COVID-19: the viral replicative phase and the hyperinflammatory phase. The latter is the most severe phase of the disease, manifesting as extra-pulmonary systemic hyperinflammation syndrome. Coupled with the disease's inhibition of the adaptive immune response including a reduction in helper, suppressor, and regulatory T cell counts, the cytokine storm seems to be the culprit in transforming a COVID-19 infection into a COVID-19 death.

Studies revealed that in hospitalized COVID-19 patients, 33% developed Acute Respiratory Distress Syndrome (ARDS) and when this occurred there was a 45% mortality rate. ARDS can be precipitated by a hyperinflammatory response to a wide range of lung insults, including SARS-CoV-2, other pathogens, sepsis, pneumonia, aspiration, and inhalation injury. Of ICU admissions for COVID-19 patients approximately 75% had developed ARDS at some time during their admission. Diagram (6) reveals the mechanisms activated during ARDS

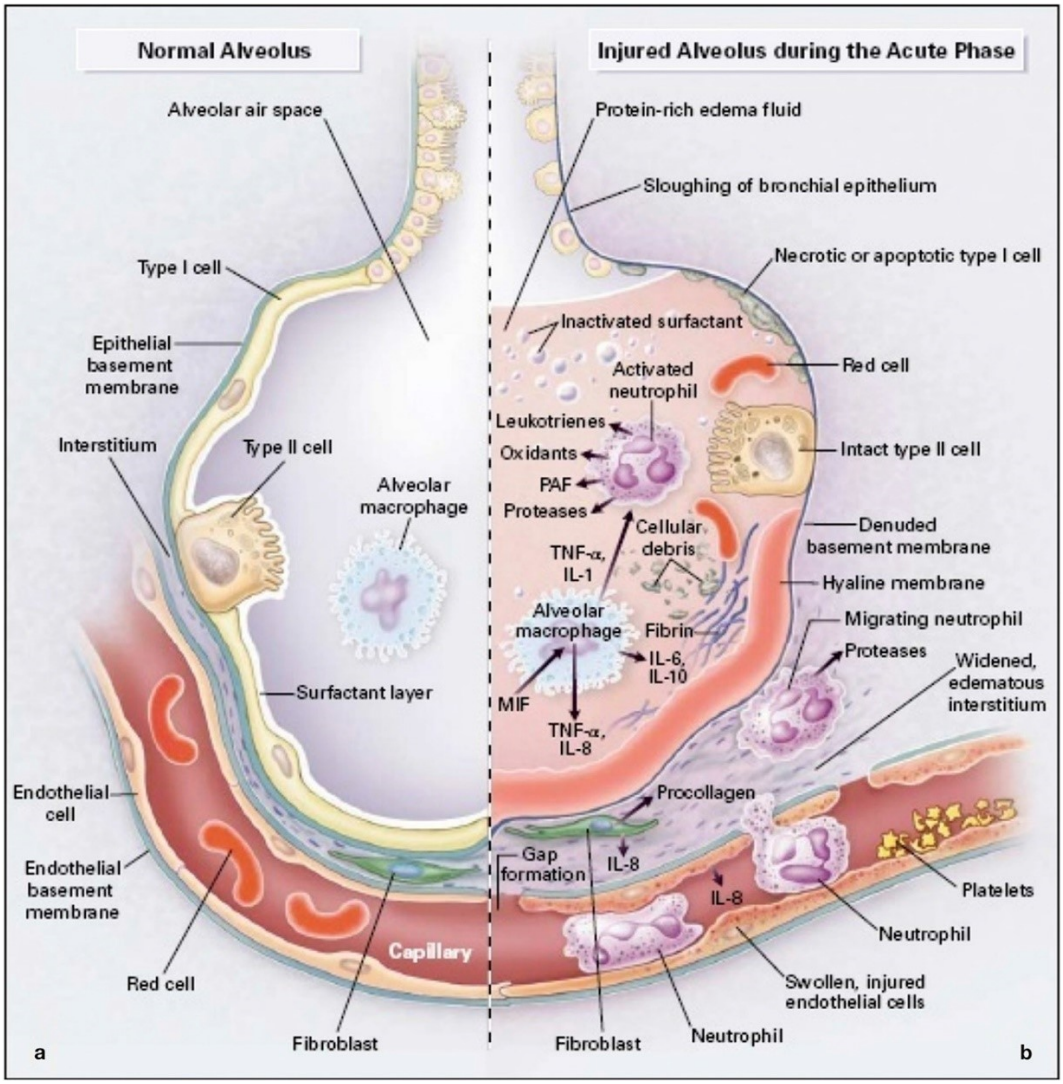


Diagram (6); ARDS Mechanism Model

It was also found that in hospitalized COVID-19 patients, 46% developed some degree of Acute Kidney Injury (AKI: a sudden and temporary loss of kidney function), and when this occurred there was found to have an associated 50% mortality rate. AKI is responsible for inflammatory and oxidative stress mechanisms that damage multiple other organs, including the lungs, heart, liver, intestines, and brain. Inflammatory cytokines are among the biomarkers implicated in high morbidity and mortality in patients with AKI. The studies also revealed that 19% of these patients having developed AKI required dialysis. Additionally, renal involvement in chronic respiratory diseases is commonly observed in clinical practice and several studies have reported that prevalence of renal failure is higher in patients with diseases affecting mainly the lungs.

IV. INFLAMMATORY CAUSES TO OTHER MEDICAL CONDITIONS

Various medical conditions are caused, exacerbated, and/or characterized by unwanted inflammation. Infections, such as bacterial, viral, and fungal infections; trauma, such as from falls, automobile accidents, gun and knife wounds; cardiovascular events, such as aneurysms and ischemic events often associated with surgery; and endogenous inflammatory reactions, such as pancreatitis and nephritis, often lead to profound dysfunction of the homeostatic mechanisms involved in regulating cardiovascular and immune system function. Several of these conditions, such as ischemia and infections, through abnormal or excessive activation of the immune system, may result in cardiovascular dysfunction that can develop over a period of hours to days, and which, under certain circumstances, can be life threatening or even fatal.

Certain cell types are critical to the dysfunction of the cardiovascular and immune systems. For example, leukocytes, especially neutrophils, contribute to the pathogenesis and progression of various inflammatory conditions, including systemic inflammatory response syndrome (SIRS), sepsis, ischemia/reperfusion injury and acute respiratory distress syndrome (ARDS). In addition, activated platelets enhance leukocyte adhesion and promote leukocyte activation. While inflammation and a systemic immune response can be beneficial in certain circumstances, they can also be fatal.

An “activated leukocyte” is understood to mean a leukocyte that, in response to a challenge, for example, when exposed to an endotoxin (e.g., lipopolysaccharide), has an enhanced ability to elicit an immune response relative to a leukocyte that has not been challenged. For example, an activated neutrophil (PMN), is a neutrophil that, in response to a challenge, for example, when exposed to an endotoxin (e.g., lipopolysaccharide), has an enhanced ability to migrate, phagocytose, and produce an oxidative burst response relative to a neutrophil that has not been challenged. Activation can also be determined via an up-regulation of cell surface CD11b. An activated monocyte is a monocyte that, in response to a challenge, for example, when exposed to an endotoxin (e.g., lipopolysaccharide), has an enhanced ability to release cytokines relative to a monocyte that has not been challenged. An “activated platelet” is understood to mean a platelet that, in response to a challenge, for example, when exposed to an endotoxin (e.g., lipopolysaccharide), becomes adherent to other platelets, to leukocytes, and to certain proteins, for example, coagulation factors. Platelet activation can be quantified by determining the percentage of circulating monocytes that have platelets adhered to their cell surface. Activated leukocytes also include primed

leukocytes. For example, a primed neutrophil (PMN), is a neutrophil that, in response to a challenge, for example, when exposed to an endotoxin (e.g., lipopolysaccharide), has an enhanced ability to undergo an oxidative burst response relative to a neutrophil that has not been challenged.

When a leukocyte is activated, selectins are produced by the leukocyte. This altered selectin production can facilitate binding between the leukocyte and other leukocytes. In turn, the binding between leukocytes can increase selectin production in the additionally bound leukocytes, yielding exponential binding of leukocytes. Thus, selectins may be useful to enhance sequestration. Proteins, protein complexes, and/or protein components known to bind leukocytes include CD11a, CD11b, CD11c, CD18, CD29, CD34, CD44, CD49d, CD54, podocalyxin, endomucin, glycosaminoglycan cell adhesion molecule-1 (GlyCAM-1), mucosal addressing cell adhesion molecule-1 (MAdCAM-1), E-selectin, L-selectin, P-selectin, cutaneous lymphocyte antigen (CLA), P-selectin glycoprotein ligand 1 (PSGL-1), leukocyte functional antigen-1 (LFA-1), Mac-1, leukocyte surface antigen p150,95, leukocyte integrin CR4, very late antigen-4 (VLA-4), lymphocyte Peyer's patch adhesion molecule-1 (LPAM-1), intracellular adhesion molecule-1 (ICAM-1), intracellular adhesion molecule-2 (ICAM-2), intracellular adhesion molecule-3 (ICAM-3), inactivated C3b (C3bi), fibrinogen, fibronectin, peripheral lymph node addressing (PNAd), endothelial vascular adhesion protein 1 (VAP-1), fractalkine, CCL19, CCL21, CCL25, and CCL27. Other large molecules known to bind leukocytes include hyaluronic acid, glycosaminoglycans (GAGs), and fucosylated oligosaccharides and their precursors.

Agents used to adhere with platelets include one or more of glycoprotein Ibα (GPIbα), glycoprotein IIb (GPIIb), glycoprotein IIIa (GPIIIa), CD41, CD61, von Willebrand Factor, β.sub.2-integrin macrophage antigen-1, selectins such as P-selectin, and a cell-adhesion molecule.

Inflammatory injury in organs can result in microvascular damage induced by leukocyte activation and aggregation, as well as platelet activation and aggregation. These activated cells can contribute to microvascular stasis and reperfusion injury by releasing toxic compounds into a patient's tissue. In acute inflammation, activated leukocytes and platelets interact as a gel-like structure within the vessel. This leads to poor perfusion of the tissue, which normally is supplied with oxygen and nutrients by the capillaries. Activated leukocytes additionally cause damage by extravasating across the endothelium into the tissue, where they release toxic agents normally intended to destroy invading microbes or clear out necrotic debris. Activated platelets additionally cause damage by enhancing the activation and endothelial transmigration of leukocytes. When these processes are not controlled, they can lead to tissue injury and death.

Systemic Inflammatory Response Syndrome (SIRS) is the thirteenth leading cause of death in the United States of America. Severe sepsis with SIRS occurs in 200,000 patients annually in the U.S. with a mortality rate of 30-40%, even with use of intensive care units and broad-spectrum antibiotics. SIRS is diagnosed largely on observed physiological changes such as increase (fever) or decrease (hypothermia) in body temperature, increased heart rate (tachycardia), increased respiration rate (tachypnea), elevated or diminished white blood cell counts, and inadequate perfusion of tissues and organs. A decrease in blood pressure is a complication associated with SIRS that occurs late in the course of the syndrome. Specifically, a decrease in blood pressure can reflect the development of shock and contribute to multiple organ failure, which is a leading cause of death in these patients. Septic shock is a condition that includes the clinical observations of the presence of an infection and a drop in blood pressure despite fluid resuscitation and proper cardiac blood output. A similar condition, sepsis syndrome, includes similar physiological signals with no evidence of any type of infection. Other insults, which induce a sepsis-like condition include pancreatitis, burns, ischemia, multiple trauma and tissue injury (often due to surgeries and transplants), hemorrhagic shock and immune-mediated organ dysfunction.

The standard therapies for SIRS and septic shock involve administration of antibiotics to bring the infection under control and fluid/colloid therapy to maintain circulating blood volume. Frequently, drugs that help maintain blood pressure, such as dopamine and vasopressin, are also administered.

Cardiopulmonary bypass (CPB) can induce SIRS, activating complement and coagulation systems and stimulating cytokine production. A large number of therapeutic approaches are under investigation to limit the activation and accumulation of leukocytes during CPB. In fact, animal and early clinical data suggest amelioration of lung and kidney damage during CPB surgery with the use of leukocyte capture and manipulation. There remains a need for improved treatments of inflammatory conditions, such as cardiovascular shock, sepsis, systemic inflammatory response syndrome and anaphylaxis.

Methods for treating and/or preventing inflammatory conditions by cytopheretic extracorporeal sequestration of leukocytes and/or platelets and inhibiting or deactivating their inflammatory action have been studied and may be promising yet invasive usually requiring a filtration device connected to a cardiac bypass system or a dialysis portal. The sequestered cells can therefore, in theory, be deactivated and/or their release of pro-inflammatory substances inhibited.

NOTE: There are no current corporeal means and methods for such cell sequestration, manipulation, and return to circulation. If a corporeal methodology can be developed not having to sequester the specific leukocytes and or platelets

yet treat them in such a manner as to inhibit the release of the pro-inflammatory substance or to deactivate the leukocyte and/or the platelet a paradigm shift treatment modality would be provided to the Healthcare System and millions of lives could be saved. One method may expose the pulmonary parenchyma and circulation to biological or chemical techniques that instead of sequestering cells, use ‘nano cells’, biomolecules (for example, proteins or nucleic acids), or other small molecules to cause the leukocyte and or platelet to respond to activation in a preferred manner. In this method the positive responses would be encouraged while the negative immune responses would be discouraged. In one petitioned aspect, these nano cells or one or more of the above listed agents that promote cellular adhesion attach to the leukocyte and platelets as with normal respiration and oxygenation of the blood. Once circulating, this act of cell adhesion and ‘internal sequestration’ promotes the intended beneficial response and during the normal course of hemoglobin dissociation and CO2 production, the nano cells may be preferably affected by CO2 and are then either exhausted and or caused to be eliminated with the CO2 expiration in the lungs in one or more respiratory cycles.

It is also believed that calcium chelators, for example, citrate, lead to a low Ca.sub.i environment thereby inhibiting release of a pro-inflammatory substance from the leukocytes and/or deactivating the leukocytes. Pro-inflammatory substances may include destructive enzymes and/or cytokines from the leukocytes. This inhibition and/or deactivation leads to an amelioration of the inflammatory state of the leukocytes. In this way, the proposed mechanism adheres to leukocytes, for example, neutrophils and monocytes, and inhibits release of a pro-inflammatory substance from the leukocytes and/or deactivates the leukocytes, for example, with citrate and/or a low-Ca.sub.i environment. The pseudo-sequestration and inhibition and/or deactivation of platelets can be achieved in a similar fashion.

The Bio-Atmosphere is not limited to a particular type or kind of leukocyte inhibiting agent. Leukocyte inhibiting agents include, for example, anti-inflammatory biological agents, anti-inflammatory small molecules, anti-inflammatory drugs, anti-inflammatory cells, and anti-inflammatory membranes. In some embodiments, the leukocyte inhibiting agent is any material or compound capable of inhibiting activated leukocyte activity including, but not limited to, non-steroidal anti-inflammatory drugs (NSAIDs), anti-cytokines, imatinib mesylate, sorafenib, sunitinib malate, anti-chemokines, immunosuppressant agents, serine leukocyte inhibitors, nitric oxide, polymorphonuclear leukocyte inhibitor factor, secretory leukocyte inhibitor, and calcium chelating agents. Examples of calcium chelating agents include, but are not limited to, citrate, sodium hexametaphosphate, ethylene diamine tetra-acetic acid (EDTA), triethylene tetramine, diethylene triamine, o-phenanthroline, oxalic acid and the like. The leukocyte inhibiting agent can be any protein or peptide known to inhibit leukocytes or immune cells including, but not limited to, angiogenin, Complement Factor D, the disulfide C39-

C92 containing tryptic angiogenin fragment and synthetic homologs of the same; the agent also can be those proteins, peptides, and homologs reported by Tschesche et al. (1994) J. BIOL. CHEM. 269(48): 30274-80, Horl et al. (1990) PNAS USA 87: 6353-57, Takashi et al. (2006) AM. J. RESPIRAT. CELL AND MOLEC. BIOL. 34: 647-652, and Balke et al. (1995) FEBS LETTERS 371: 300-302, that may facilitate inhibition of release of a pro-inflammatory substance from a leukocyte and/or deactivate a leukocyte. Moreover, the leukocyte inhibiting agent can be any nucleic acid known to inhibit release of a pro-inflammatory substance from the leukocyte and/or deactivate the leukocyte. The leukocyte inhibiting agent can be in solution or lyophilized. Additionally, the inhibition of release of a pro-inflammatory substance from a leukocyte and/or deactivation of a leukocyte may occur temporally before, during, and/or after adhesion of the preferred agent(s) to the leukocyte.

It should also be understood that the deactivation techniques described herein also can apply to platelets. Agents used to deactivate a platelet and/or inhibit release of a pro-inflammatory substance from a platelet include, but are not limited to, agents that inhibit thrombin, antithrombin III, meglatran, herudin, Protein C and Tissue Factor Pathway Inhibitor. In addition, some leukocyte inhibiting agents can act as platelet inhibiting agents. For example, calcium chelating agents, such as citrate, sodium hexametaphosphate, ethylene diamine tetra-acetic acid (EDTA), triethylene tetramine, diethylene triamine, o-phenanthroline, and oxalic acid can deactivate a platelet and/or inhibit release of a pro-inflammatory substance from a platelet.

Although the extracorporeal sequestering devices have patent protection with their specific extracorporeal process, we do not believe the Bio-Atmosphere and methodology pertains to any “methods known in the art”. It is this exact intent and explicit description provided of which Vertu Realities has petitioned the novel therapeutic deliver system and is now developing the Bio-Atmosphere Therapeutic Delivery System components of the BioZone System and investigating the mitigating agents and methods applicable to the intent.

The Bio-Atmosphere method may additionally be applicable for treating individuals having or at risk of developing an inflammatory condition. The inflammatory condition is optionally selected from the group consisting of systemic inflammatory response syndrome (SIRS), polyarteritis, Wegener's granulomatosis, autoimmune vasculitis, anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, extracorporeal membrane oxygenation (ECMO), cardiopulmonary bypass syndrome, acute respiratory distress syndrome (ARDS), acute lung injury (ALI), chronic obstructive pulmonary disease (COPD), sepsis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, multiple sclerosis (MS), psoriasis, allograft rejection, asthma, acute renal failure, chronic renal failure (CRF), end stage renal disease (ESRD), cardiorenal syndrome

(CRS), chronic heart failure (CHF), stroke, myocardial infarction (MI), hepatorenal syndrome, cirrhosis of the liver, diabetes mellitus (type 2 diabetes), and acute organ failure from ischemic reperfusion injury to myocardium, central nervous system, liver, kidney, or pancreas.

Additionally, the BioZone Bio-Atmosphere's potential countermeasure to inflammatory responses, cytokine storm prevention, tissue protection, and even tissue repair is one application, now being proposed for the Aerospace Industry as a potential modulator and mitigator to Radiation Induced Lung Injuries (RILI) and the negative consequences of lung injuries propagating to additional organ damage as described above. Additional positive measures applicable of the Bio-Atmosphere to the Aerospace Industry include countering the negative gravity circulatory volume redistribution which leads to visual dysfunction, changes in the cerebrospinal fluid volume and intracranial pressure, alterations in the anatomical structures of glands such as the Pituitary Gland, enlargement of the ventricles of the brain which may produce a hydrocephalic condition, disturbances in the vestibular system of the inner ear leading to vertigo and or nausea, to name a few examples.

V. COVID-19

The following discussion pertains to the pathogenicity of viral contagions upon exposure to lung tissue in humans and more specifically, as experienced by the COVID-19 Coronavirus. We will first define the background anatomy and physiology of human lung tissue cells identifying the cellular receptor functions and responses. Next we will address the specifics of the SARS 2 Covid-19 Coronavirus infection and the potential areas of intervention.

BACKGROUND

Lung Receptor Anatomy and Biology

In order to dive further into the benefits of the BioZone's Isolation Bio-atmosphere treatment for Covid-19 patients, it is imperative to have knowledge of the basic systems that are involved. Covid-19 and atomized therapeutics utilize separate systems, and it is important to understand each systems' function to grasp the correlation. We will see that not only are the systems targeted in individual manners, but that the systems themselves are interconnected in their biological pathway responses.

First, we will look at three receptor systems and their functions in the lungs.

Beta-Adrenoceptors in Lung Tissue

One of the more common receptor systems found throughout the lung's tissue is the beta adrenoceptors. These receptors are highly concentrated throughout the entirety of the smooth muscle tissue in the upper and lower airways. The density of the receptors in airway smooth muscle does not change at different airway levels, so that bronchioles have a similar density to large airways. This shows that regardless of location in the lung system, there are beta adrenoceptors present. In addition to their high concentration, they are also broken down into a few main subtypes. The two subtypes of beta receptors are beta1 and beta 2, and studies show that 70% of the beta-adrenoceptors in the lungs are beta 2. This subtype is located in smooth muscle, epithelium, and submucosal glands while beta1 receptors are located primarily in the submucosal glands. The two subtypes are also located in the alveolar wall. There is a uniform distribution of beta-receptors on the alveolar wall with a ratio of beta1: beta 2 receptors of 2:1. While beta 2-receptors are primarily responsible for the regulation of the bronchial constriction response, the beta 1 receptors work on regulating mucus production in the alveoli. The mechanism by which the beta 2-receptor causes smooth muscle relaxation is the activation of beta 2- adrenoceptors on airway smooth muscle (ASM) generates intracellular cAMP by adenylate cyclase activation. This in turn activates its effector molecules, cAMP-dependent protein kinase A, which is a Rap1 guanine nucleotide exchange factor. The cAMP wave also downregulates Rho and causes calcium to be sequestered in the smooth endoplasmic reticulum. This in combination with the phosphorylation of regulatory proteins leads to airway smooth muscle relaxation in the tissue. This effect translates into other forms as the more common beta 2- receptor is located in more areas than just lung tissues.

Alternate Functions for Beta2-Adrenoceptors

In addition to lung tissue, other systems of the body possess these same receptor types. Most prevalent being the white blood cells of immune system. While our bodies are well equipped to fight viruses and other pathogens that may enter our system, the side effects of the immune response can be debilitating for a patient. The key result of white blood cells fighting off an infection is the inflammatory response caused in the body. The beta 2-adrenoceptors in the ASM are also found on these immune system cells. The inflammatory response generated from by white blood cells can be downregulated via stimulation of the beta 2-receptors. Peter Barnes claims, "Inflammatory cells that are involved in asthma and COPD, including eosinophils, neutrophils, T lymphocytes, and macrophages, all express a

low number of beta 2-receptors. In vitro, beta 2-agonists have been shown to inhibit the release of inflammatory mediators from these cells". Here we can see that these receptors have a similar function in white blood cells as with ASM. This process of de-inflammation leads to a cascade of other benefits, one being a reduction in plasma exudation. Plasma exudation refers to the leaking of plasma out of postcapillary venules into the 4-surrounding space. This is usually the result of acute inflammation in tissues. Just as in white blood cells, beta 2-receptors are also located on the endothelial cells in these post-capillary venules. Stimulation of these receptors helps to prevent the leaking of fluid and edema in nearby systems. The reactions that stimulate the beta-adrenoceptor system are crucial when taking into account treatment options for lung diseases. In addition to this receptor system, another exists that has important cross correlation to the beta-adrenoceptors.

Muscarinic Acetylcholine Receptors

Another key receptor system in the lungs is the muscarinic acetylcholine receptor system. This receptor type is typically associated with its function in the parasympathetic nervous system. Airway smooth muscles receive parasympathetic input from the vagus nerve, and the activation of the vagus causes smooth muscle contraction. The substrate for these receptor types is acetylcholine (ACh), and its binding to its target receptor can lead to this negative symptom and impaired lung function. Acetylcholine (ACh) is the neurotransmitter in the parasympathetic nervous system, both in the ganglia and at the neuroeffector junction. ACh activates muscarinic acetylcholine receptors postsynapse on ASM and mucous glands to elicit bronchoconstriction and mucous secretion, respectively. As with the beta-adrenoceptors, the mAChRs are broken down into a few subtypes. The main subtypes are M1, M2, and M3 mAChRs. The receptor most related to the ASM constriction is the M3 mAChRs. When ACh binds to an M3 receptor in the lungs, phospholipase C will generate the second messenger IP3. This leads to a calcium release from the sarcoplasmic reticulum and the contraction of ASM via calcium-calmodulin dependent myosin light chain kinase. The other receptor types use the same substrate but serve slightly different functions. The M2 receptor subtypes are also referred to as inhibitory muscarinic receptors or auto receptors. These subtypes are mainly located in the presynaptic terminals of the post ganglionic parasympathetic neuron while some can be located directly on the ASM. On the post-ganglionic nerve, they inhibit the release of ACh from the nerve to the ASM. When these receptors bind ACh they reduce the release ACh on to other muscarinic receptors in the ASM. However, receptors on the ASM serve a slightly different function. These receptors inhibit relaxation induced both by β-adrenoreceptor agonists and adenylyl cyclase activation with forskolin. Thus, M2 receptors contribute to smooth muscle contraction by functionally antagonizing

Gαs-induced relaxation. In the ASM the M2 receptors promote the constriction of the airway smooth by blocking other methods of dilation. The final receptor subtype is the M1 subtype. These, like the M2, are located in two different locations. First, they are in the postsynaptic terminals of the post ganglionic parasympathetic nerve. In this position they serve to help stimulate neurotransmission. If this receptor were blocked so that ACh could not bind, there would be a possible reduction in neurotransmission of the vagus nerve signal. This would reduce the amount of ACh released onto the ASM and reduce the constriction response in ASM. Second, M1 subtypes are also found in the airway submucosal glands along with M3 subtypes. In these glands, ACh binding causes both subtypes to secrete mucins and fluid. In submucosal glands, muscarinic receptors are found on both serous cells that secrete fluid and mucous cells that secrete mucins. Both M1 and M3 receptors are present in human and animal submucosal glands. Given the variation in subtypes and their functions, it is also important to note the methods by which the substrate, ACh, is presented to the receptor.

Stimulation Methods in Muscarinic Receptors

With the multiple subtypes of receptors, their specific location in the lung determines how they will be stimulated. There are two manners in which the mAChRs are stimulated with ACh. The first is stimulation through cholinergic nerves and the parasympathetic nervous system. However, the level of innervation of the nerve system changes throughout the lung tissues.
Postganglionic fibers then innervate airway smooth muscle and submucosal glands. Vagal innervation of the airways is predominantly in large airway and diminishes peripherally with no motor innervation of small airway and lung parenchyma. In addition, the concentration mAChRs begins to decrease into the smaller airways of the lungs. Given this decrease in the nervous systems ability to stimulate, another method of ACh delivery occurs. The ACh needed to activate the mAChRs can also be produced from other cells in the tissues of the lungs. This is done by choline acetyltransferase (ChAT). Both epithelial and inflammatory cells possess transferase enzymes, and enzyme production increases as the cell is exposed to inflammatory factors such as tumor necrosis factor alpha (TNF-alpha). These two pathways are how bronchoconstriction occurs via this receptor type. They work in unison to alert the body to stress and infection no matter which part of the lungs are affected. While both these receptor systems are different, they are also strongly interconnected.

Muscarinic and Beta-Adrenoceptor Cross Correlations

The correlation between both receptor systems serves as a building block in the treatment of lung diseases. Multiple connection points in their individual pathways lead to cross-stimulation and inhibition. Both of these receptor pathways work to stimulate second messengers in the cell. The muscarinic system works to increase intracellular calcium and activate protein kinase C (PKC). These events promote contraction of ASM via the calcium-calmodulin dependent myosin light chain. On the other hand, the beta-adrenoceptor system works to promote the formation of the second messenger cAMP and promote the relaxation of ASM. The activation of PKC serves the function of desensitizing the beta 2-receptor. Given that the beta 2-receptor is a G-protein coupled receptor, PKC will phosphorylate its Gs subunit leading to a desensitization of the receptor. This reduces the beta adrenoceptor's ability to generate the second messenger for relaxation via its downstream pathway. On the contrary, the beta-adrenoceptor pathway is shown to lower the concentration of intracellular calcium via its sequestration caused by cAMP. The cAMP-dependent protein kinase A (PKA) activated by the adrenoceptor pathway is also thought to reduce the production of IP3, the second messenger in the ASM contraction of the muscarinic receptor system. In addition, the stimulation of the beta adrenoceptors leads to the prevention of release of ACh into the synaptic space by activating calcium-activated potassium channels in the postganglionic neuron. It is likely that these channels reduce the concentration of Ca2+ by hyperpolarizing the cell membrane and, consequently, inhibit the release of ACh. These different inhibition relationships show how one system effects another to alter the response of lung tissues. The final receptor system to take into consideration is the one that SARS-CoV-2 works on directly.

The Angiotensin Receptor System

The renin-angiotensin system (RAS) is commonly categorized as a system for inflammation and de-inflammation. The system utilizes the combined system of converting enzymes and angiotensin. These enzymes are expressed predominately in the cardiovascular and pulmonary systems. Renin is released from the kidneys in response to a low sodium intake and sympathetic stimulation. This leads to the formation of angiotensin peptides that serve various functions. The main focus is placed on angiotensin II (AngII). When renin leads to the formation of different angiotensin peptides, angiotensin converting enzyme (ACE) converts the local angiotensin one (AngI) into its more detrimental form, AngII. An increase in AngII is associated with inflammation in tissues and an elevated vascular constriction. Another converting enzyme receptor, angiotensin converting enzyme two (ACEII), breaks down AngII and cleaves this angiotensin into angiotensin 1-7. With AngI, ACEII breaks this down into angiotensin 1-9. This breakdown of the angiotensin

proteins promotes vasodilation and de-inflammation. This system is important in understanding how the SARS-CoV-2 infects a host.

SARS-Covid-19 Coronavirus

For the past few years, the COVID-19 pandemic has been the focal point in healthcare and research. This disease has permanently changed daily life and left a historic impact on the world. Most people have felt the effects of this pandemic either directly, via infection, or indirectly, via change in workflow, financial impact, etc. The main question for this virus still remains today; how do we treat this illness effectively? While many ideas are being tested and suggested, a definite answer has yet to be procured. Vaccine rates are climbing on a daily basis, serving as the first and most beneficial form of prevention when it comes to spread and severity of infection. However, as new variants continue to mutate into existence, we are left wondering how long this virus will be an active threat.

For this purpose, novel treatments must be added to the oral and intravenous pharmaceutical vaccine and drug armamentarium that seems to be losing efficacy with every new viral mutation. Additionally, the expected decrease in viral lethality with continued mutation has been brought into question with the latest MBB, BQ.1 and BQ.1.1 Covid strains seemingly being more dangerous than the predecessor Omicron strains. Are we to return to lockdowns and mandating healthcare providers to work in nearly intolerable working conditions donning and doffing PPE all day long?

The BioZone Intensive Care Unit (BICU) is under development to provide the urgently needed contagion isolation enclosure wherein direct patient care may be provided. The need for PPE garments to prevent provider exposure as well as contamination of the treatment work zones from viral dissemination can now been mitigated with the BICU provisions.

SARS-CoV-2 In the Lungs

Next, we explore the process by which SARS-CoV-2 enters the body and begins the process of infection. SARS-CoV-2's detrimental effects can send patients to the hospital and even cause fatality. So, what are the key issues with a COVID diagnosis and what symptoms do we need to treat to protect patient health? Transmission is important for understanding how this process starts.

SARS-CoV-2 Transmission and Deposition

Most individuals understand that SARS-CoV-2 is transmitted via aerosol and the air. The term aerosol is defined as solid particles or liquid droplets with small diameters of about a few nanometers and micrometers. SARS-CoV-2 has been found to spread quickly from person to person via this method. This is because aerosols released from individuals begin to spread out as they travel outwards. This results in the intake of the virus through inhalation. Some of the main methods of transmission from an infected host are coughing, sneezing, breathing and speech. These actions produce aerosol particle of various sizes. An article by Zuo et al. makes an estimate to the number of virions in an aerosol. It states, "COVID-19 patient sputum contains 106 −1011 viral RNAs per milliliter, although this number "can overestimate infectious virions". If sputum matter is aerosolized as 5 μm particles, then the average number of virions per aerosol, using 1011 virions/mL, is no greater than 50". They go on to state that the vast majority of the aerosols, approximately 67%, released from an infected patient contain at least one active virion if not more. The various sizes and properties of these individual particles play a role in the virus's deposition into the lungs. A smaller particle size will allow for better sedimentation into the lungs. The particle size affects what area of the lungs the virus will be located in. SARS-CoV-2 location in the lungs is based on the size of the particle the virion travels in and can be lodged in the alveoli, large airways or small airways. Regardless of the location of the infection, the process by which SARS-CoV-2 enters the lungs tissue is the same.

SARS-CoV-2 Infection Process

The renin-angiotensin system (RAS) serves as the entry point of SARS-CoV-2 into the body. The SARS-CoV-2 virus uses its spike protein to latch onto a target receptor in the lung tissue after inhalation. The spike protein has a strong binding affinity to the ACEII receptor of the RAS system. It uses this receptor to gain entry into the lungs cells and begin replication. While the virus may be localized to any point of the lung system, and ACE receptor will more than likely be present near that location in an epithelial cell. As SARS-CoV-2 enters the cell, it begins to pass through its replication cycle and use the host cells machinery to create more active virions. After lysing the cell, the virions spread to other areas of the lungs and other cells. As this process continues, more sections of lung tissue become infected with the virus and the replication process increases exponentially. The virus moves from the airways deeper into the tissues of the lungs. This creates a rapid onset of infection once the process beings. SARS-CoV-2's infection process is a double edge sword. When the virus uses an ACEII for entry, it consequently lowers the expression of ACEII via the RAS pathway activation. This increase is AngII results in system inflammation and constriction. The spread of SARS-CoV-2 also leads to alveolar infection which is where the most severe symptoms begin.

Symptoms of Infection

As the infection of SARS-CoV-2 moves through the lung system, at some point it reaches the alveolus. The type II pulmonary alveolar epithelial cells are some of the main cells that express ACEII the most, leaving them a prime target for SARS-CoV-2 entry. As this area becomes infected, problems develop with breathing function and oxygen exchange. The issues produced by COVID-19 are compounded in nature. Not all patients will find a way into the hospital from a SARS-CoV-2 infection, but the magnitude of damage and symptoms is the final determinate. The first systematic response is the activation of multiple white blood cell types. From mast cells to neutrophils, the innate immune system activates to defend the body from the invading virus. This storm leads to intense inflammation in the lungs. An article in BioEssays by Natesan Vasanthakumar gives light to this by stating, “Cytokine storm and hyperinflammation occurs in COVID-19 irrespective of lymphopenia. Considering the cytokine 14 storm and potential activation of the NLRP3 inflammasome, it is likely that immune hyperactivation occurs in COVID-19”. One of these cytokines is tumor necrosis factor alpha (TNF-a). As mentioned before this inflammation factor is used as a signaling molecule in the muscarinic acetylcholine system. This leads to an increase mucus production and bronchoconstriction in SARS-CoV-2 infected patients. Increased mucus aggregation has been noticed in the distal airways and alveoli in the postmortem study of COVID-19 patients. The mucus production is also increased by the presence of white blood cells which work to produce ACh via their ChAT. This viral infection of the alveolus also produces a breakdown of the epithelial cell structure. This allows for the fluid secretion in the interspace between the post capillary venules and edema in the alveolus. It also forms cellular debris build up that prevents proper airway exchange. Oxygen exchange and saturation rates drop as more alveoli become infected and damaged. The combination of sluffing cells, mucus, fluid, and constriction can be devastating for an infected patient. This complexing issue can lead some more severely infected COVID patients into acute respiratory distress syndrome (ARDS). Approximately 5% of COVID-19 patients progress to ARDS. This is made worse by the downgraded expression of the ACEII by SARS-CoV-2 in its infection process. ACEII has been shown to help prevent the progression into ARDS by its anti-inflammatory effects. This risk of ARDS results in a high level of concern in regard to mortality. Many patients who develop ARDS end up being placed on a ventilator to keep them breathing. A treatment option must be one that supports a decrease in mucus production, fluid secretion, cytokine storm and bronchoconstriction. An answer to these problems is provided by the Bio-Atmosphere approach therapeutic delivery system.

Diagram (7) is an artist rendering of the activation mechanism that the Covid-19 SARS Coronavirus initiates leading to the parenchymal destruction and debris

accumulation in the lung tissue. Additionally shown are two therapeutic approaches intended to block the harmful consequences of the cellular activation process.

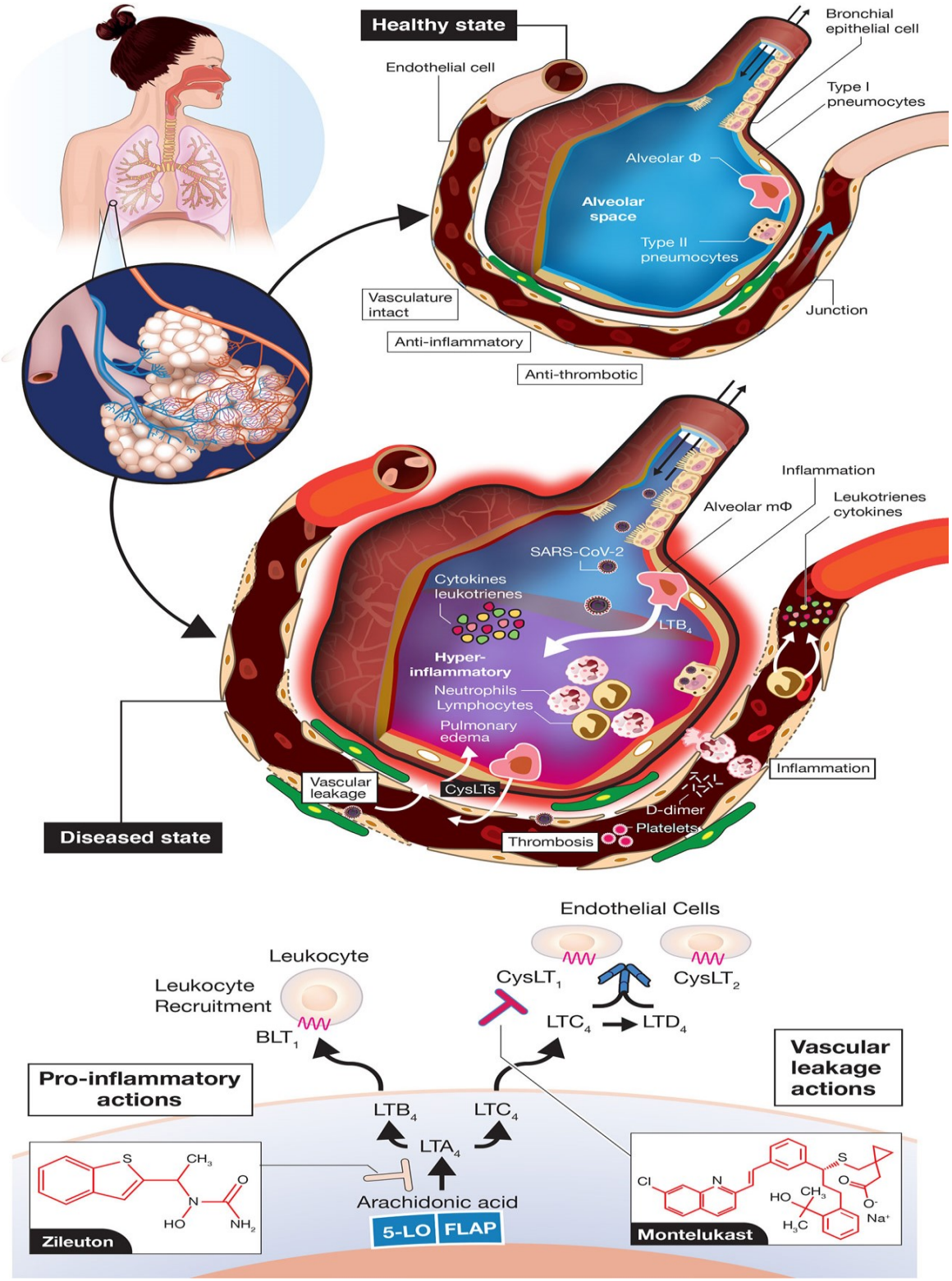


Diagram (7); Covid-19 Activation of Immune Processes

VI. RADIATION EXPOSURE

Radiation exposure in space has been extensively studied yet there continues to exist the need for additional countermeasures if we are going to safely travel prolonged space missions such as going to Mars. In this discussion we will allude to the radioprotector, mitigator, and treatment provisions to combat radiation exposure syndromes. A complete gamut of deterrents and therapies will be needed for once the spaceship travels beyond the earth's magnetosphere then the astronauts lives are in jeopardy due to acute and chronic radiation exposure. Additionally, the worst-case scenarios must be proactively mitigated prior to launch considering the longer the astronauts are in space, the higher the probability of the astronauts being exposed to a major solar particle events which will couple with cumulative galactic cosmic ray exposure, both with potential harm to the crew.

NASA has published articles identifying known radiation exposure sequelae such as general cumulative radiation carcinogenesis, adverse effects on the eyes, central nervous system, digestive tract, blood components as well as the circulatory system as a whole, premature aging, immunological suppressive disorders and degenerative disorders, for example. As of now, we are limited to administering antioxidants (radioprotectors) to hopefully reduce the damage to various organs by radiation exposure and giving some form of mitigator agent during or shortly after exposure. The determining of radiation exposure involves the use of dosimetry monitors once the exposure has occurred, and then monitoring the effects of the given exposure and radiation accumulation on the human body.

NASA reports assert "There are several mechanisms being targeted for the development of radiation countermeasures to address radiation syndromes, including the scavenging of free radicals, blocking cell death signals, facilitating repair of damaged molecules, and inducing regeneration of injured tissue. Additionally, NASA acknowledges more advanced radioprotectors and mitigators will be required for longer duration missions". According to (Ryan 2012) these may include targeted gene therapy with targets focused on the TGFβ1 pathway inhibitor, synthetic superoxide dismutase/catalase mimetics, recombinant IL-12, toll-like receptor-5 antagonist, and inhibitors of cyclin-dependent kinases.

According to one NASA article "There are several biological radiation countermeasures currently available or under development that can be investigated to determine their efficacy in treating acute radiation syndrome due to solar particle events. Several new therapies are also being explored, many of which are already in early-stage clinical trials, to evaluate their toxicity and safety as space radiation countermeasures. Mechanistic studies of possible biochemical routes for

countermeasure actions must be combined with approaches to extrapolate model system results to humans for such countermeasures to be used operationally by NASA. It will be important moving forward to bear in mind that the efficacy of any biological countermeasure will need to be determined under the appropriate dose and space radiation environment, and the impact on other risk areas must be considered. Selecting effective radioprotectors or mitigators will also involve practical concerns, such as ease of administration, effectiveness period, impact on performance, side effects, toxicity, shelf-life, and drug interactions, all of which will be factored into the adoption of any biological countermeasure. Continued surveillance of new technologies and radioprotectors/mitigators will guide the identification and validation of appropriate biological countermeasures for long-duration space missions".

Background and Objective

Radiation Induced Lung Injury (RILI) has been thoroughly investigated due to the inadvertent consequences of radiation therapy use to address cancer treatment. We postulate that a prolonged exposure to ionizing radiation from space travel will subject the astronaut to a similar pathophysiologic pathway of pneumonitis followed by pulmonary fibrosis. The occurrence of RILI will greatly affect the prognosis and quality of life of astronauts during a prolonged space mission or upon return to home. A cascade of subsequent health issues will ensue accordingly which could potentially negatively affect the astronaut's ability to carry out their mission duties or lead to premature death.

The main objective of this narrative review is to describe the available evidence concerning RILI, from the biological mechanism to the clinical management. The underlying causes of RILI are multifactorial, including gene-level changes, the influence of signaling pathways, the convergence of various cells, as well as the expression of cytokines and chemokines. Based on the various mechanisms of RILI, the BioZone Project intends to introduce novel treatment strategies.

RILI is a constantly developing and changing process including radiation pneumonitis and radiation lung fibrosis. Different kinds of inflammatory and immune cells such as macrophages, fibroblasts, and T cells play key roles in the development of RILI and transforming growth factor-β (TGF-β), interleukin-4 (IL-4), IL-13, and interferon-γ (IFN-γ) are also participants in this process.

We recognize the damage in lung tissue as radiation-induced lung injury (RILI), which occurs due to exposure to ionizing radiation, leading to pathophysiological and interstitial changes on imaging.

RILI can be divided into two stages: radiation pneumonia (RP) in the early stage, and radiation lung fibrosis (RLF) in the later stages. This is marked by a series of pathophysiological changes, including epithelial and endothelial cell damage, infiltration of inflammatory cells, release of cytokines, differentiation of fibroblasts, deposition of extracellular matrix (ECM), and synthesis of collagen. These processes ultimately lead to imaging and clinical changes. On the molecular level, these changes correspond to a series of non-specific symptoms including cough, weakness, dyspnea, fever, abnormal imaging, and others. Thus, research into treatments and prevention of these diseases is crucial.

After irradiation, tissues are damaged either directly or indirectly. Ionizing radiation leads to direct deoxyribonucleic acid (DNA) damage, including base deletion, DNA single-strand break, and DNA double-strand break. The bulk of injuries are repaired through cellular repair functions, and residual damaged cells are induced to apoptosis or mutation, which may contribute to tumor development.

Another indirect damage of DNA is activated by reactive oxygen species (ROS), which are primarily produced by mitochondrial oxidative metabolism to participate in the growth, differentiation, progression, and death of cells in the biological process. Furthermore, water molecules of irradiated cells are ionized to generate an excessive number of ROS, including superoxide, hydrogen peroxide, hydroxyl radicals, and nitrogen species (NGS), which indirectly cause DNA damage. Except for analogous nuclear DNA damage with direct impacts, ROS also induces mitochondrial DNA damage, which leads to protein carbonylation, lipid peroxidation, increased oxidative metabolism, and enhanced rates of spontaneous gene mutations and neoplastic transformation. Moreover, DNA damage and ROS activation stimulate series of intracellular signaling pathways and signal factors including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and interleukin 1 (IL-1).

In the lung tissue, ROS generation is derived from endothelial cells, neutrophils, eosinophils, alveolar macrophages, and alveolar epithelial cells (AECs). ROS generation causes damage to the endothelial barrier, as well as an increase in vascular permeability and trans-endothelial migration of enormous leukocytes.

The development of RILI is divided into various stages by different scholars. Arroyo-Hernández *et al.* distributed RILI to five phases based on the molecular changes: early phase, latent phase, exudative phase, intermediate phase, and fibrotic phase

In the early stage (RP), damage-associated molecular pattern molecules (DAMPs) are released from cells to recruit many immune effector cells to accumulate the damage of lung tissue and contribute to tissue remodeling. Under the induction of intercellular cell adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), neutrophils and macrophages arrive one

after another and release IL-3, IL-6, IL-7, TNF-α, and TGF-β to produce an inflammatory reaction. In the late stage (RLF), T helper 2 (Th2) cells participate in the profibrotic process. Neutrophils and macrophages induced profibrotic effects via the secretion of TGF-β, IL-6, and PDGF. Blood monocytes are recruited to lungs and differentiate into fibroblasts and myofibroblasts. Neutrophils secrete elastase and matrix metalloproteinases to contribution to accumulation ECM. Finally, pulmonary fibrosis occurs.

As mentioned above, the human alveolar epithelium consists of type-I and II pneumocytes, which make up 90% and 10% of cells in the alveoli, respectively. Ionizing radiation induces direct DNA damage as well as the generation of reactive oxygen (RO), which contribute to the apoptosis of the sensitive type-I pneumocytes within minutes. As the precursors of type-I cells, type-II pneumocytes increase and drive re-epithelialization of the alveolus. Moreover, ionization of water molecules generates ROS. ROS also causes edema of the alveolar walls, increased vascular permeability, and exudation of proteins into the alveolar space, which further reduces the alveolar septa. The process of epithelial and endothelial cell damage manifests as damage to the connections between cells, accompanied by impaired regulation of myofibroblasts and deposition of excessive ECM. A large number of blood exudate and inflammatory cells accumulate in the alveolar cavity, which leads to intra-alveolar edema and aggregates numerous fibroblasts and induces their differentiation into myofibroblasts. Activated myofibroblasts then secrete angiotensin and hydrogen peroxide, which in turn induce AECs apoptosis. The following cytokines are released during this process: tumor necrosis factor-α (TNF-α), IL-1, IL-6, high-molecular-weight mucin-like antigen KL-6, PDGF-β, and basic fibroblastic growth factor.

Macrophages and fibroblasts are activated under the inducements of ILs, TNF, TGF, and PDGF to synthetize ECM. Macrophages are divided into two types, including classic-activated macrophages (M1), which is regulated by Th1-derived cytokine IFN-γ, and bypass-activated macrophages (M2), which is regulated by Th2-derived cytokines IL-4 and IL-13. Macrophages have been implicated in carcinogenesis, angiogenic switch, local invasion, and metastasis. In the early stage of RILI, M1 macrophages secrete pro-inflammatory cytokines to induce inflammation and produce massive ROS (via a ROS-induced cascade) to further impair lung tissue. In the late stage of RILI, M2 macrophages secrete profibrotic cytokines to promote the development of RLF. M2 macrophages express Arg-1, which controls L-proline production that is required for collagen synthesis by activated myofibroblasts to induce mesenchymal transition of epithelial cells. The TGF-β secretion of M2 macrophages has a similar effect. These cytokines promote radiation-induced fibrosis in the late stage.

Under the action of chemokines, various types of inflammatory cells, such as lymphocytes, macrophages, neutrophils, and mononuclear cells, proliferate,

aggregate, and migrate into the pulmonary interstitial tissue, release various cytokines, and cause alveolar edema, eventually lead to the occurrence of pulmonary fibrosis. After radiation, lung tissue produces massive fibroblasts and myofibroblasts, causing extensive production of collagen, infiltration of inflammatory cells, and remodeling of the ECM. Fibrosis of the alveolar septum subsequently leads to extensive occlusion of the alveoli.

VII. DIAGNOSING RILI

RILI being divided into two stages gives us: RP in the early stage and RLF in the late stage. RP most frequently occurs within 1–6 months, while the onset of RLF occurs as a late toxicity beyond 6 months. There are non-specific symptoms that occur in both stages: fever, chest pain, cough, fatigue, tachypnea, and others. Patients may express aggravation of original respiratory symptoms and/or new clinical manifestations. For example, the frequency and degree of cough and phlegm increases with severe symptoms, such as hypoxemia and newly occurred respiratory failure. Physical examination may include pleural friction rub or moist rales. Laboratory testing sometimes reveals a high percentage of neutrophils. Most relevant, imaging classically demonstrates findings most typically consist of development of a diffuse a ground-glass opacity or mass-like consolidation in the field of radiation treatment, with bronchial pull and scar-like changes. RLF has similar clinical symptoms and physical examination, but fever is rare. Imaging cross-sectional imaging classically shows ventilated bronchial signs, strip shadows, lung consolidation shadows, or honeycomb changes within the irradiated lung tissue. RP is often accompanied by bacterial, viral, or fungal infections, such as Pneumocystis carinii while RILI is treated according to the grade conditions.

In conclusion, the pathological change of RILI is mediated by a series of cells and cytokines. Radiation induces DNA damage, and the generation of reactive oxygen stimulates the activation of inflammatory pathways. Direct damage to epithelial alveolar and endothelial cells leads to pro-inflammatory and pro-fibroblast activity. The release of a variety of cytokines drives the action of macrophages and the differentiation of fibroblasts. Th1 and Th2 cells participate in the polarization and infiltration of cytokines. TGF-β plays a key role in this process, and IFN-γ, macrophages, and PGE2 have a positive impact in inhibiting lung fibrosis. It is therefore established that a variety of chemokines promote the development of RILI and Viral Induced Pneumonitis of which both are being addressed as treatable entities with the novel BioZone System’s Bio-Atmosphere therapies titled the BioFlow and BioNeb.

VIII. PATHOPHYSIOLOGY OF ISCHEMIC INJURIES

In the last five decades since Closed Chest CPR (CC-CPR) was first introduced many studies have been published documenting its ineffectiveness (i.e., survival rates under 20%) in maintaining cerebral viability in cases of cardiac arrest both in the hospital and in the field. Indeed, there is evidence that the survival rate of patients experiencing in-hospital cardiac arrest has declined since Closed Chest CPR replaced Open Chest CPR (OC-CPR) in the 1960's. In the fifty years since its implementation there has never been a formal, organized assessment of the utility of this technique in terms of cost vs. benefit either financially or medically.

In patients who survive following resuscitation with Closed Chest CPR, the incidence of both transient and permanent neurological deficits and reduced quality of life are high.

In recent years there has been a growing awareness of the inadequacy of Closed Chest CPR, with a call by some to return to Open Chest CPR and vigorous research by others to optimize CC-CPR to address the dismal survival rates and usually poor neurological outcome. Increasingly, public healthcare policy is coming to reflect the reality that neurologists, cardiologists and intensivists have long understood: "CC-CPR doesn't work.". This is reflected in the recent policy change by the American Red Cross, wherein bystanders to cardiac arrest patients are now urged to activate the Emergency Medical System (EMS) first and start CPR second, instead of the other way around. This change reflects a growing awareness that CC-CPR is largely ineffective and that a patient's best chance for recovery is early defibrillation and associated definitive therapy.

This may seem an extreme statement, particularly to those who have not witnessed the all-too-common tableaux played out in intensive care units around the world of the brain dead or vegetative cardiac arrest victim consuming tens of thousands of dollars in medical resources.

The staggering cost of CC-CPR in teaching, healthcare, and patient/family emotional and financial resources when weighed against the dubious benefit suggests that society might have been better served if the CC-CPR program had never been implemented. The conclusion seems inescapable that CC-CPR is most effective at producing individuals who either are brain dead, or in a persistent vegetative state.

The problem with CC-CPR (or any in-field resuscitation technique) is Cerebral Ischemia. While mechanical or other device-oriented means of optimizing CC-

CPR may well be developed, and the first-response use of defibrillators may become more commonplace, the fundamental problem of ischemic time before restoration of adequate circulation remains.

For many of the 325,000 persons in the United States who will experience sudden cardiac death (SCD) in the coming year, there will be little or no possibility of rescue. Cardiac arrest will occur without warning, often in situations not conducive to activation of the EMS. However, for many of those patients, there will have been a warning that they are at increased risk of SCD. A prior myocardial infarct (MI), familial history of arrhythmic disease, or iatrogenic risk such as CABG or angioplasty, will often provide ample warning that SCD could occur. In MI alone the incidence of SCD within the first year following infarct is 14%. The development of more sophisticated markers for SCD in post MI patients, such as increased R-R interval regularity, is also making it possible to identify with increasing accuracy those who are at risk of SCD.

What can be done to improve the disappointing overall success rate of CPR? Does increasing the ability to identify patients at risk for SCD offer the possibility of therapeutic interventions such as anti-arrhythmic drugs and implantable defibrillators? Is there some way to pre-medicate or pre-treat patients who are at risk to increase their chances of surviving an ischemic episode with intact mentation?

A review of the literature in experimental cerebral resuscitation and the pathophysiology of cerebral ischemia (CI) suggests a number of approaches using both pre- and post-insult medication which may provide protection against cerebral ischemia for those at risk for SCD and which have acceptable costs and risk-to-benefit ratios.

While a wide range of post-insult interventions are currently being investigated in animal and clinical trials, relatively little attention has been paid to the possibility of pre-medication of the at-risk population combined with post-insult therapy. Additionally, despite almost universal agreement that CI is a multifactorial insult, there has been little or no research aimed at developing a multimodal method of managing the multiple insults and compromises to brain metabolism that are known to occur.

Before suggestions are put forth for prevention and/or amelioration of ischemic injury, it is desirable to briefly review the requirements for adequate cerebral perfusion and the basic mechanisms of cerebral ischemic injury as they are currently understood.

Requirements For Adequate Cerebral Perfusion

Normal cerebral blood flow (CBF) in man is typically in the range of 45-50 ml/min/100g between a mean arterial pressure (MAP) of 60 and 130 mmHg. When CBF falls below 20 to 30 ml/min/100g, marked disturbances in brain metabolism begin to occur, such as water and electrolyte shifts and regional areas of the cerebral cortex experience failed perfusion. At blood flow rates below 10 ml/min/100g, sudden depolarization of the neurons occurs with rapid loss of intracellular potassium to the extracellular space.

The Mean Arterial Pressure (MAP) necessary for cerebral viability following extended resuscitation efforts in dogs has been found to be above 40 mm Hg. It has been speculated that a minimum MAP of 45 to 50 mm Hg is required to preserve cerebral viability in man.

Unfortunately, as is now well documented, conventional CC-CPR being generally incapable of consistently delivering MAPs much above 30 mm Hg in man. A clinical evaluation of manual and mechanical CPR (using a pneumatically driven chest compressor and ventilator) demonstrated that only 3 of 15 acute cardiac arrest patients presenting for emergency room resuscitation had MAPs above 40 mm Hg.

It should be emphasized that these studies evaluated a highly selected patient population, where the underlying cause of cardiac arrest was primary cardiac failure without other organ system failure, dehydration, sepsis, or pulmonary hypoxia as an underlying cause.

Quite often, the patient presenting for cryopreservation suffers from a variety of pathologies which can be expected to further reduce the ability of closed chest CPR to deliver adequate MAP or adequate arterial blood oxygenation (paO2). Pneumonia, pulmonary and systemic edema, hemorrhage, sepsis, liver failure, space-occupying lesions of the lungs, and a host of other pathologies can all compromise gas exchange and reduce vascular tone and circulating blood volume. Even in the patient experiencing optimum machine-delivered CPR, lung compliance and blood gases tend to deteriorate rapidly during CPR, perhaps as a result of pulmonary edema secondary to high intrathoracic venous pressures.

As the foregoing analysis makes clear, many, if not most, cryopreservation patients will suffer significant periods of cerebral anoxia, ischemia, or hypoperfusion before they receive more effective cardiopulmonary support such as OC-CPR, extracorporeal circulation utilizing a membrane or bubble oxygenator, or high impulse CPR.

Mechanisms of Ischemic Injury

Early observations on the mechanisms of ischemic injury focused on relatively simple biochemical and physiological changes which were known to result from interruption of circulation. Examples of these changes are loss of high-energy compounds, acidosis due to anaerobic generation of lactate, and no reflow due to swelling of astrocytes with compression of brain capillaries. Subsequent research has shown the problem to be far more complex than was previously thought, involving the action and interaction of many factors.

Biochemical Events

Within 20 seconds of interruption of blood flow to the mammalian brain under conditions of normothermia, the EEG disappears, probably as a result of the failure of high-energy metabolism. Within 5 minutes, high-energy phosphate levels have virtually disappeared (ATP depletion) and profound disturbances in cell electrolyte balance start to occur: potassium begins to leak rapidly from the intracellular compartment and sodium and calcium begin to enter the cells. Sodium influx results in a marked increase in cellular water content, particularly in the astrocytes.

Calcium

Normally, calcium is present in the extracellular milieu at a concentration 10,000 times greater than the intracellular concentration. This 10,000:1 differential is maintained by at least the following four mechanisms: (1) active extrusion of calcium from the cell by an ATP-driven membrane pump; (2) exchange of calcium for sodium at the cell membrane driven by the intracellular to extracellular differential in the concentration of Na+ as a result of the cell membrane's Na+ — K+ pump, (3) sequestration of intracellular calcium in the endoplasmic reticulum by an ATP-driven process, and (4) accumulation of intracellular calcium by oxidation-dependent calcium sequestration inside the mitochondria.

The loss of cellular high-energy compounds during ischemia causing the loss of the Na+ - K+ gradient, virtually eliminates three of the four mechanisms of cellular calcium homeostasis. This, in turn, causes a massive and rapid influx of calcium into the cell. Mitochondrial sequestration, the remaining mechanism, causes overloading of the mitochondria with calcium and diminished capacity for oxidative phosphorylation. Elevated intracellular Ca++ activates membrane phospholipases and protein kinases. A consequence of phospholipase activation is the production of free fatty acids (FFA's) including the potent prostaglandin

inducer, arachidonic acid (AA). The degradation of the membrane by phospholipases almost certainly damages membrane integrity, further reducing the efficiency of calcium pumping and leading to further calcium overload and a failure to regulate intracellular calcium levels following the ischemic episode. Additionally, FFAs almost certainly have other degradative effects on cell membranes.

The production of AA as a result of FFA release causes a biochemical cascade ending with the production of thromboxane and leukotrienes. Both these compounds are profound tissue irritants which can cause platelet aggregation, clotting, vasospasm, and edema, with resultant further compromise to restoration of adequate cerebral perfusion upon restoration of blood flow.

Free Radicals

During ischemia, the hydrolysis of ATP via AMP leads to an accumulation of hypoxanthine. Increased intracellular calcium enhances the conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO). Upon reperfusion and reintroduction of oxygen, XO may produce superoxide and xanthine from hypoxanthine and oxygen. Even more damaging free radicals could conceivably be produced by the metal catalyzed Haber-Weiss reaction as follows:

O2- + H2O —-Fe3 ——> O2 + OH-+ OH-

Iron, the transition metal needed to drive this reaction, is present in abundant quantities in bound form in living systems in the form of cytochromes, transferrin, hemoglobin and others. Anaerobic conditions have long been known to release such normally bound iron. Indirect experimental confirmation of the role of free iron in generating free-radical injury has come from a number of studies which have confirmed the presence of free-radical breakdown products such as conjugated dienes and low molecular weight species of iron.

During reperfusion and re-oxygenation, significantly increased levels of several free-radical species that degrade cell and capillary membranes have been postulated: 1) O2-, OH-, and free lipid radicals (FLRs). O2- may be formed by the previously described actions of XO and/or by release from neutrophils which have been activated by leukotrienes.

Re-oxygenation also restores ATP levels, and this may in turn allow active uptake of calcium by the mitochondria, resulting in massive calcium overload and destruction of the mitochondria.

Mitochondrial Dysfunction

Calcium loading and free-radical generation are no doubt major contributors to the mitochondrial ultrastructural changes which are known to occur following cerebral ischemia. In addition to the structural alterations observed, there are biochemical derangements such as a marked decrease in adenine nucleotide translocase and oxidative phosphorylation. There is also an accumulation of FFAs, long-chain acyl-CoA, and long-chain carnitines. Of these alterations, the accumulation of long-chain acyl-CoA is perhaps most significant, since intramitochondrial accumulation of long-chain acyl-CoA is known to be deleterious to many different mitochondrial enzyme systems.

Lactic Acidosis

While it is clearly not the sole or even the major source of injury in ischemia, lactic acidosis does apparently contribute to the pathophysiology of ischemia. It has been shown, for instance, that lactate levels above a threshold of 18 – 25 micromol/g result in currently irreversible neuronal injury.

Decrease in pH as a consequence of lactic acidosis has been shown to injure and inactivate mitochondria. Lactic acid degradation of NADH (which is needed for ATP synthesis) may also interfere with adequate recovery of ATP levels post ischemic. Lactic acid can also increase iron decompartmentalization, thus increasing the amount of free-radical mediated injury.

Excitotoxins

A rapidly growing body of evidence indicates that excitatory neurotransmitters, which are released during ischemia, play an important role in the etiology of neuronal ischemic injury. Those areas of the brain which show the most “selective vulnerability” to ischemia, such as the neocortex and hippocampus, are richly endowed with excitatory AMPA (alpha-amino-hydroxy-5-methyl-4-isoxazole proprionic acid) and NMDA (N-methyl-d-aspartate receptors).

Initially there was much optimism that blockade of the NMDA receptor would provide protection against delayed neuronal death following global cerebral ischemia. The use of NMDA receptor blocking drugs has shown significant promise in ameliorating focal cerebral ischemic injury; a number of studies have

demonstrated marked reduction in the severity of ischemic injury to focal areas (particularly the poorly perfused “penumbra” surrounding the no-flow area) as a result of treatment with glutamate-blocking drugs such as dextrorophan or the experimental anticonvulsant MK-801. In vitro studies with cultured neurons have demonstrated that excitatory neurotransmitters cause neuronal injury and death even in the absence of hypoxic or ischemic injury. In vivo studies have confirmed a massive release of glutamate and aspartate during both regional and global cerebral ischemia.

In regional or focal cerebral ischemic injury, the NMDA receptor remains activated for a long period due to the prolonged interval of poor perfusion in the area at the edges of the infarct (the “penumbra”). However, in complete or global ischemia there is good resumption of blood flow following restoration of circulation with prompt uptake of glutamate and aspartate and resultant relatively rapid inactivation of the NMDA receptors. Another factor limiting the role of the NMDA receptor in mediating injury in global cerebral ischemia may be the rapid and pronounced drop in pH which occurs in global as opposed to focal ischemia, since low pH is known to inactivate the NMDA receptor. These reasons are probably why NMDA receptor inhibitors have not proved effective in preventing global cerebral ischemic injury. Recently, attention has turned to non-NMDA antagonists such as inhibitors of the Kainate and AMPA receptors.

The mechanisms by which excitotoxins cause cell injury is not yet fully understood. It is known that they facilitate calcium entry into neurons. However, these agents are neurotoxic even in cell culture where the medium is calcium free. In the case of Kainate and AMPA receptor activation, the likely mode of injury is sensitization of the CA1 pyramidal cells during ischemia such that when normal signaling is restored at the end of the ischemic insult, and normal intensity input from the Schaffer collaterals is resumed, lethal cell injury results, perhaps from abnormal calcium regulation in the CA1 cells or other metabolic derangements not yet understood.

Neutrophil Activation

Since the late 1960s, polymorphonuclear leukocytes (PMNLs) and monocytes/macrophages have been implicated as significant causes of pathology in cerebral ischemia. During the last decades there has been a veritable explosion of research documenting the role of PMNLs in reperfusion injury. Most of the initial work done in this area focused on PMNL-mediated reperfusion injury to the myocardium, establishing that PMNL activation and subsequent plugging and degranulation (resulting in release of oxidizing compounds) is responsible for the no-reflow phenomenon following myocardial ischemia. In particular, the work of

Engler has demonstrated that PMNL activation is responsible for plugging at least 27% of myocardial capillaries and is further responsible for the development of edema and arrhythmias upon reperfusion.

To what extent leukocyte plugging occurs in the brain following global cerebral ischemia remains controversial. Anderson, et al. have examined the question of how rapidly leukocyte plugging occurs following cerebral ischemia using a bilateral carotid artery plus hypotension model in the dog. They noted no leukocyte plugging after 3 hours of reperfusion following a 40-minute ischemic episode.

However, it is clear from a growing body of work that neutrophils are a major mediator of ischemic injury in a variety of organ systems and that their acute activation is responsible for many of the effects of ischemia observed in the brain and other body tissues, including the loss of capillary integrity and the degradation of ultrastructure upon reperfusion.

When PMNLs are activated they generate large amounts of hydrogen peroxide. A large fraction of the hydrogen peroxide, aided by myeloperoxide (also released by activated PMNLs), reacts with the halides Cl-, Br-, or I- to produce their corresponding hypohalous acids (HOX). Because the concentration of Cl- is more than a thousand times greater than the other halides, the hydrogen peroxide-myeloperoxidase system probably generates Cl- most often in the form of HOCl. HOCl is more commonly known as household bleach and is capable of damaging a wide range of organic molecules including most of those that make up the structure of the cells and proteinaceous extracellular matrix. As Klebanoff has pointed out, the amounts of HOCl generated by the neutrophil are awesome: 106 neutrophils can generate 2 x 107 mol of HOCl – enough to destroy 150 million E. Coli in a matter of milliseconds.

However, the direct destructive effects of HOCl are probably limited in vivo by a variety of mechanisms. Most probably the hypohalous acids act to inflict the lion's share of injury by interacting with PMNL, collagenase, elastase, gelatinase, and other proteinases. As is shown in the diagram below, it is now believed that the oxidants released from the neutrophil create a halo of oxidized alpha-1-proteinase inhibitor that allows released elastase (and probably others of the 20 or so known neutrophil-secreted proteolytic enzymes) to begin degrading the extracellular matrix, thus destroying capillary integrity and interfering with tissue metabolism and anabolism.

In complete circulatory arrest, it is clear that neutrophil activation with accompanying release of HOCl and activation of elastase is a key factor in initiating the systemic cascade of inflammation/immune response which terminates in delayed multisystem organ failure. The extent to which this pathway is a factor in acute global cerebral ischemic injury in cardiac arrest is not yet clear.

Hypoperfusion Following Reperfusion

An apparently significant contributor to reperfusion injury is hypoperfusion after restoration of spontaneous circulation. The work of Hossman, et al, and Sterz, et al, has demonstrated the critical importance of providing adequate circulatory support following global cerebral ischemia. Loss of autonomic regulation depressed myocardial function secondary to ischemic insult of the myocardium, and autonomic dysfunction all serve to depress MAP and cerebral perfusion following restoration of circulation. Both Hossman's and Sterz's work has demonstrated significant improvements in neurological outcome if circulation is supported both extracorporeally and/or with pressors during reperfusion.

Histological Ultrastructural Change

Ischemic changes in cell architecture begin almost as rapidly as ischemic changes in biochemistry. Within seconds of the onset of cerebral ischemia, brain interstitial space almost completely disappears. Loss of interstitial space is a consequence of cell swelling secondary to sodium influx and failure of membrane ionic regulation. There have been several studies of the ultrastructural alterations associated with prolonged global cerebral ischemia. Notable is the work of Kalimo et al in the cat, as well as Karlsson and Schultz, and Van Nimwegen, et al in the rat. These investigators describe the following changes in common in these animals' brain ultrastructure after varying periods of global cerebral ischemia (GCI):

1) Changes At 10 Minutes

After 10 minutes of GCI, a significant number of cells (but not all) show clumping of nuclear chromatin and a modest increase in electron lucency (probably due to dilution of the cytosol by extracellular fluid). After 30 minutes, further changes include increased cytoplasmic swelling (particularly in the astrocytes), swelling and shape change of the mitochondria, and some loss of mitochondrial matrix density. Microtubules disappear and there is detachment of the ribosomes from the cisternae of the endoplasmic reticulum. There is also disassociation of the polyribosomes, and single ribosomes lose their compact structure with associated failure of protein synthesis. Of note is the stability of the lysosomes over this time course.

2) Changes At 60 Minutes

After 60 minutes of GCI, the above changes have become more pronounced with more conspicuous swelling of the ER cisternae. The mitochondria begin to show

slight inner matrix swelling and occasional flocculent densities (probably due to accumulated calcium).

3) Changes At 120 Minutes

After 120 minutes of GCI, the changes discussed above are more pronounced and a larger number of mitochondria exhibit the presence of flocculent densities evidencing calcium overload which is currently considered irreversible. Published electron micrographs reveal intact lysosomes and seem to confirm other studies which indicate that lysosomal rupture and subsequent catastrophic autolysis is not a feature of early (1 – 4 hours) ischemic injury.

From a cryonics (i.e., information-theoretic perspective), it is important to point out that throughout even a 120-minute-period of normothermic cerebral ischemia, the appearance of the plasma membrane layers, including synapses and myelin sheaths, is only altered modestly. Indeed, the first ultrastructural changes associated with what is currently considered lethal cell injury are to the mitochondria and ribosomes, and these do not usually appear until after 30 minutes of GCI.

At least one study of post-mortem ultrastructural degradation has been conducted on a small number of human subjects. The histological and ultrastructural changes experienced in patients with 25 to 85 minutes of GCI, and without extensive pre-mortem brain trauma or pre-mortem cerebral no-reflow of prolonged duration, closely parallel those observed in animal models of GCI: astrocytic edema, clumping of nuclear chromatin, disassociation of the polyribosomes, detachment of the ribosomes from the ER cisternae, and swelling of the mitochondria with the presence of flocculent densities. Stability of the lysosomes and conservation of the structure of the neuropil over this time-course are well documented.

Opportunities For Intervention

With the understanding of the mechanisms of the pathophysiology of cerebral ischemia having evolved to the point outlined above, many possible interventions suggest themselves. Indeed, the literature of cerebral resuscitation is a vast one and is growing rapidly with the release of papers exploring a variety of monomodal approaches to treating cerebral injury secondary to both global and regional ischemic insults.

However, despite the widely held belief that cerebral ischemic injury is multifactorial in nature, there has been almost no work done examining multimodal methods of treatment. There is also almost a complete absence of

studies which address the potential of pre-treatment in ameliorating cerebral ischemic injury, particularly pretreatment with nonproprietary agents such as antioxidant nutrients. This kind of approach is of particular importance to the cryonics community where a significant number of patients present for cryopreservation in a slow failure mode that allows for active intervention.

The approach to protecting cryopreservation patients against cerebral ischemic injury outlined in this text is a multimodal approach which address the following known sources of cerebral ischemic injury:

1) Numerous studies have suggested a cerebro-protective effect for a variety of calcium channel blockers administered post-insult.

2) Free radical damage: Free radicals have long been understood to be a major source of cerebral ischemic pathology. Similarly, there have been a number of studies which suggest that free radical associated ischemic injury can be reduced greatly or eliminated by pre- or post-insult treatment with nutritional antioxidants such as vitamin E, selenium, vitamin C, and beta carotene. Theoretical considerations also suggest other possible therapeutic agents such as those known to elevate neuronal (intracellular) glutathione levels for protection from cerebral ischemic injury.

3) Phospholipase activation has been implicated as a significant source of injury in both cold and warm ischemia. The phospholipase inhibitor quinacrine has reduced cold ischemic injury in an organ preservation model as well as myocardial reperfusion injury. Quinacrine may be effective in attenuating normothermic cerebral ischemic injury as well.

4) The importance of mitochondrial dysfunction in preventing recovery following global cerebral ischemia has been demonstrated in a recent study by Rosenthal, et al. They demonstrated the effectiveness of acetyl-l-carnitine in improving both neurological function and normalizing brain high energy metabolism in the dog following 10 minutes of normothermic cardiac arrest.

5) Protection against the deleterious effects of excitotoxicity has been addressed in a number of ways, including the use of both NMDA and Kainate receptor inhibiting drugs. As has been previously discussed, excitotoxicity is clearly a significant source of reperfusion injury and must be addressed in any multimodal therapeutic approach to cerebral ischemia.

6) As was previously noted, extracorporeal perfusion to support MAP, facilitate reperfusion through initial hypertension, ensure adequacy of cerebral perfusion, and allow for induction of mild hypothermia have been shown to be beneficial in achieving a favorable outcome following 10-to-12-minute periods of global cerebral ischemia.

7) Inhibition of the inflammatory cascade and the adhesion and degranulation of polymorphonuclear lymphocytes by both drug treatment and by their removal via filtration have been shown to lessen reperfusion injury in the lungs and heart. As a consequence, they presumably lessen the likelihood of development of the post resuscitation syndrome, at least in extracerebral tissues.

Summary

As the foregoing has hopefully made clear, neuronal ischemic changes occur rapidly with significant structural changes being observed over a time-course of minutes rather than hours. The significance of these changes in terms of damage to identity-critical structures (i.e., those encoding memory and personality) is not currently known since we do not yet understand how memory is encoded, or more generally, which brain structures (gross or ultrastructural) are critical to mentation.

As a consequence of our ignorance about what structures need to be preserved, it is our opinion that a very conservative approach to cryopreservation patient transport should be followed. In practice, what this means is that every reasonable effort should be made to minimize cerebral ischemic injury. Achieving a reasonable cost versus benefit tradeoff in actual practice will naturally be a matter of some debate. An attempt has been made in the development of this protocol to strike a reasonable balance between cost and complexity and anticipated benefit to the patient. A fairly conservative approach has been used in the application of new technologies without a proven track record of clinical success in cerebral resuscitation.

The fields of cerebral resuscitation and cryonics have existed long enough to have observed a number of “fads” and “hot new techniques” come and go. An attempt has been made here to apply only those research modalities which have shown promise in a number of researchers’ hands, and whenever possible, to have in-house verification of the effectiveness of these modalities.

A schematic demonstrating the various microvascular and cellular pathophysiologic consequences which occur during the primary and secondary injury in hypoxic ischemic brain injury (HIBI). Decreased cerebral oxygen delivery manifests as reduced neuronal aerobic metabolism, causing reduced cellular adenosine triphosphate (ATP) production. Intracellular calcium accumulation leads to mitochondrial toxicity and further reduced ATP production. Inability to sustain cellular respiration results in cell death and apoptosis. Additionally, in the microvasculature, endothelial dysfunction leads to a porous blood-brain barrier, formation of cerebral edema, formation of microthrombi and

limitation of cerebral blood flow with exacerbation of cellular ischemia. *AQP 4* Aquaporin-4, *RBC* Red blood cells, *WBC* White blood cells.

IX. MECHANISMS of CELL DEATH in HEART DISEASE

The major cardiac syndromes, myocardial infarction and heart failure, are responsible for a large portion of deaths worldwide. Genetic and pharmacological manipulations indicate that cell death is an important component in the pathogenesis of both diseases. Cells die primarily by apoptosis or necrosis, and autophagy has been associated with cell death. Apoptosis has long been recognized as a highly regulated process. Recent data indicate that a significant subset of necrotic deaths is also programmed. In our review, we discuss the molecular mechanisms that underlie these forms of cell death and their interconnections. The possibility is raised that small molecules aimed at inhibiting cell death may provide novel therapies for these common and lethal heart syndromes.

Introduction

Cells die primarily by apoptosis or necrosis. Apoptosis is a highly regulated mode of cell suicide. Although necrosis has traditionally been regarded as passive and unregulated, data accumulated over the past decade indicate that a substantial proportion of necrotic deaths is actively executed by the cell in a highly regulated manner. This form of necrosis is sometimes referred to as regulated or programmed. Both apoptosis and necrosis play critical roles in normal biology including prenatal development and postnatal homeostasis. Accordingly, when increased, decreased, or mis-localized, cell death plays major roles in human diseases, including cardiovascular disease, cancer, diabetes mellitus, sepsis, and some neurological disorders.

Apoptosis is characterized by cell shrinkage, fragmentation into membrane-enclosed apoptotic bodies, and phagocytosis of these corpses by macrophages, or occasionally, neighboring cells. When this clean-up operation is efficient, inflammation is avoided. ATP levels in apoptotic cells are reasonably well maintained both because of continued production and decreased expenditures. The net result of apoptosis is the stealth deletion of individual cells within a tissue. In contrast, necrosis is characterized by loss of plasma membrane integrity, cellular and organellar swelling, and marked inflammation. ATP levels are dramatically

reduced in necrotic cells, both because of severe mitochondrial damage that cripples ATP generation as well as unrestrained energy expenditures. The chicken-and-egg relationships between ATP deficits and loss of plasma membrane integrity remain unclear. Similarly, although it is tempting to speculate that the decision of a doomed cell to undergo apoptosis versus necrosis is determined by energetics, this possibility has not yet been definitively established.

Mechanisms of Cell Death

Apoptosis and necrosis are mediated by distinct, but highly overlapping central pathways (Fig.(1)). The extrinsic pathway involves cell surface death receptors (DRs) and the intrinsic pathway uses the mitochondria and endoplasmic reticulum (ER). These pathways, which mediate both apoptosis and necrosis, are linked by multiple biochemical and functional connections. Extrapolating this degree of connectivity, the possibility is raised that these cell death mechanisms comprise single unified death machinery. However, given the morphological differences among types of cell death and the presumption that each arose at a specific time in evolution for a specific purpose, the notion of a unified model remains to be established.

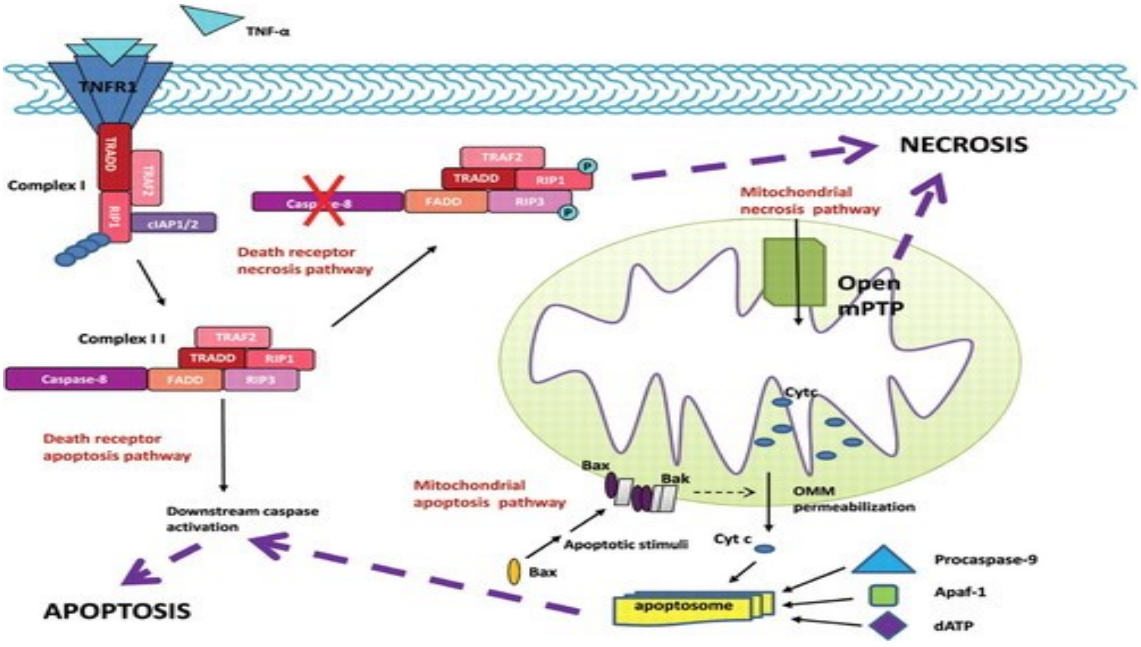


Fig. (1) Cell death pathways.

Apoptosis and necrosis are mediated by death receptor (extrinsic) and mitochondrial (intrinsic) pathways. In the death receptor pathway, a death ligand (eg, tumor necrosis factor-α [TNF-α]) binds its cognate death receptor to trigger assembly of either the death-inducing signaling complex (DISC, not shown) or complex I. When receptor interacting protein 1 (RIP1) is K63-polyubiquinated by cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2), complex I signals survival through nuclear factor-kappaB (NF-kB) activation (not shown). If death receptor dissociates from complex I, the complex is endocytosed, RIP1 undergoes deubiquitiniation, and a Fas-associated via death domain (FADD)-RIP3 complex is recruited, complex II is formed. This complex signals apoptosis or necrosis depending on procaspase-8 activity. Activation of procaspases-8 leads to cleavage and activation of downstream procaspases that proteolyze cellular proteins to bring about apoptosis. Procaspase-8 also cleaves RIP1 and RIP3, to preclude necrosis. In contrast, with caspase-8 inhibition, RIP1 and RIP3 undergo a series of cross-phosphorylation events that trigger necrosis by a variety of mechanisms. In the mitochondrial pathway, the critical event in apoptosis is permeabilization of the outer mitochondrial membrane (OMM), which results in release of mitochondrial apoptogens (eg, cytochrome c) to the cytoplasm. Complex interactions among B-cell lymphoma 2 (Bcl-2) family members (eg, Bax and Bak) mediate OMM permeabilization. Once in the cytoplasm, cytochrome c stimulates assembly of the apoptosome, a multiprotein complex in which procaspase-9 is activated. Procaspase-9 goes on to activate downstream procaspases. In contrast, the defining event in necrosis is opening of the mitochondrial permeability transition pore (mPTP) in the inner membrane, which collapses the electrical gradient across the inner mitochondrial membrane (IMM) leading to cessation of ATP synthesis and promotes the influx of water into the mitochondrial matrix resulting in severe mitochondrial swelling. Multiple connections exist between these pathways. Apaf-1 indicates apoptotic protease activating factor 1; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2 associated X protein; Cyt c, cytochrome c; TNFR1, tumor necrosis factor receptor 1; TRADD, TNF receptor-associated death domain; and TRAF2, TNFR-associated factor 2.

Extrinsic (DR) Pathway: Apoptosis and Necrosis

In the DR pathway, a variety of death ligands bind their cognate receptors to trigger cell death. Some of these ligands are soluble (eg, tumor necrosis factor [TNF]-α), and some are bound to the surface of other cells (eg, Fas ligand). The efficiency with these ligands to induce death varies with cell type. Recent work has

shown that the same death ligands may induce apoptosis or necrosis, the choice mediated by downstream events.

Binding of ligand to receptor induces the formation of either of 2 multiprotein complexes: the death-inducing signaling complex (DISC) and complex I. The DISC signals apoptosis whereas complex I can signal either apoptosis, necrosis, or cell survival. The DISC has been studied most intensively in the context of Fas ligand/Fas signaling and complex I in the setting of TNF/TNF receptor 1 signaling. However, which ligand/receptor combinations use the DISC versus complex I, or both, is incompletely understood.

In DISC formation, the binding of death ligand induces a conformational change in the cytosolic domain of the DR, which recruits an adaptor protein (eg, Fas-associated via death domain, TNF receptor-associated death domain). This adaptor protein, in turn, binds upstream procaspases-8 or -10 to form the DISC. Procaspases are the zymogen form of caspases, cystenyl proteases that cut after aspartic acid residues. Within the DISC, procaspases-8 and -10 are activated through a forced proximity mechanism. Once activated, these caspases cleave, and activate downstream procaspases-3 and -7. Caspases-3 and -7 then cut multiple cellular proteins to bring about apoptotic death through mechanisms that are incompletely understood. In most cells, activation of the extrinsic pathway alone is insufficient to kill and requires amplification through the intrinsic pathway. One means by which amplification is achieved is through the cleavage of the B-cell lymphoma 2 (Bcl-2) family protein BH3-interacting domain death agonist (Bid) by caspase-8, after which truncated Bid translocates to the mitochondria and contributes to outer mitochondrial membrane (OMM) apoptotic events described below.

In the assembly of complex I, the binding of death ligand to receptor recruits TNF receptor-associated death domain, which recruits receptor interacting protein 1 (RIP1, a serine/threonine kinase), cellular inhibitor of apoptosis proteins (IAP) 1 and 2, and TNF receptor-associated factor 2 and 5. RIP1 undergoes K63-polyubiquitination by cellular IAP 1 and 2. This provides a platform for the recruitment of additional kinases that activate nuclear factor-kappaB, resulting in the transcription of survival proteins. However, after dissociation of DR, endocytosis, deubiquitination of RIP1, and recruitment of a Fas-associated via death domain-RIP3 complex, complex I morphs into complex II. Complex II signals apoptosis when Fas-associated via death domain recruits procaspase-8 leading to its activation by forced proximity. Caspase-8 not only activates downstream caspases to bring about apoptosis, it also cleaves RIP1 and RIP3 abrogating their ability to signal necrosis. If caspase-8 activity is inhibited experimentally or by certain viral or cancer proteins, apoptosis is blocked,

obligating the cell to undergo necrosis in this pathway. Necrosis is triggered by the interaction of RIP1 with RIP3, a second serine/threonine kinase, resulting in a complex series of cross-phosphorylation events. Necrostatin-1, a small molecule inhibitor of the kinase activity of RIP1, ablates necrosis in the DR pathway.

Events in this pathway downstream of RIP1 and RIP3 are incompletely understood but include phosphorylation by RIP3 of mixed lineage kinase domain-like protein, phosphoglycerate mutase 5 (a mitochondrial phosphatase), and certain catabolic enzymes (glutamate dehydrogenase 1, glutamate ammonia ligase, and glycogen phosphorylase), the latter potentially eliciting necrosis through the generation of reactive oxygen species (ROS). The effects of ROS on the mitochondria are discussed below. In addition, ROS-mediated DNA damage leads to overactivation of poly(ADP-ribose) polymerase 1, a nuclear enzyme that consumes nicotinamide adenine dinucleotide leading to significant ATP consumption, a key feature of necrosis. Other downstream events that have been implicated in DR necrosis signaling include activation of calpains, phospholipases, lipoxygenases, and sphingomyelinases and permeabilization of lysosomes.

Intrinsic (Mitochondrial/ER) Pathway: Apoptosis and Necrosis

Mitochondria and ER are central to both apoptotic and necrotic signaling, and the intrinsic pathway mediates a more diverse array of death stimuli than does the DR pathway. These include deprivation of nutrients, oxygen, and survival factors, oxidative stress, DNA damage, proteotoxic stress, and chemical and physical toxins. Present understanding suggests that the pathways and events that mediate apoptosis and necrosis at the mitochondria are spatially and mechanistically distinct. The primary event in apoptosis is permeabilization of the OMM resulting in the release of apoptogens. In contrast, the defining event in primary necrosis is the early opening of a channel in the inner mitochondrial membrane termed the mitochondrial permeability transition pore (mPTP).

Mitochondrial Signaling: Apoptosis

The main regulators of the mitochondrial apoptosis pathway are the Bcl-2 family proteins. In addition, as discussed below, recent data also implicate these proteins in the regulation of necrosis. The Bcl-2 family is composed of both antiapoptotic (eg, Bcl-2, Bcl-extra large, Mcl-1) and proapoptotic members, and the

proapoptotics are further divided into multidomain (eg, Bcl-2 associated X protein [Bax], Bcl-2 homologous antagonist/killer [Bak]) and BH3-only proteins (multiple members). In healthy cells, Bax resides primarily in the cytosol. In response to death stimuli, Bax undergoes conformational activation, and translocates to the mitochondria, where it inserts into the OMM. Apoptotic signals also stimulate the conformational activation of Bak, which is constitutively localized to the OMM. Within the OMM, Bax and Bak homo- and hetero-oligomerize to bring about OMM permeabilization through poorly understood mechanisms. The noxious stimuli that activate Bax and Bak are transduced from various locations in the cell via specific BH3-only proteins. For example, loss of the survival signals, insulin and insulin-like growth factor 1 leads to activation of the BH3-only protein Bcl-2-associated death promoter by decreasing Bcl-2-associated death promoter phosphorylation and permitting its release from the 14-3-3 protein. The means by which BH3-only proteins activate Bax and Bak is complex. Certain BH3-only proteins called activators (eg, Bcl-2-interacting mediator, Bid) bind directly to Bax (and possibly Bak) to conformationally activate these proteins. Other BH3-only proteins called sensitizors displace the activator BH3-only proteins from antiapoptotics such as Bcl-2 and Bcl-extra large. Conversely, antiapoptotic Bcl-2 proteins inhibit Bax and Bak by sequestering the BH3-only activators, and possibly also through direct interactions with Bax and Bak.

Permeabilization of the OMM leads to the release of apoptogens, including cytochrome c, second mitochondria-derived activator of caspases/direct IAP binding protein with low isoelectric point, Omi/high temperature requirement protein A2, apoptosis-inducing factor, and endonuclease G from the mitochondria to the cytosol. Cytosolic cytochrome c and dATP bind to the adaptor protein apoptotic protease activating factor 1 resulting in a presumed conformational change that stimulates apoptotic protease activating factor 1 oligomerization and its recruitment of upstream procaspase-9 into a complex termed the apoptosome. Procaspase-9 is activated by forced proximity within this complex, and goes on to cleave and activate procaspases-3 and -7. Apoptosis is opposed by IAP family members, the same proteins that act in the DR necrosis pathway to signal survival through their K63-polyubiquination of RIP1. In the mitochondrial apoptosis pathway, these IAPs inhibit already activated downstream caspases by occluding access of substrates to the active sites of these caspases. The apoptogens second mitochondria-derived activator of caspases/direct IAP binding protein with low isoelectric point and Omi/high temperature requirement protein A2 reverse caspase inhibition by IAPs through binding to IAPs and displacing the caspases. In addition, Omi/high temperature requirement protein A2 possesses serine protease activity that cleaves X-linked IAP. Apoptosis-inducing factor, which in combination with perhaps endonuclease G causes fragmentation of DNA from ≈200 to 50 kb fragments, has been hypothesized to mediate a form of caspase-

independent cell death. However, it is possible that the primary role of apoptosis-inducing factor–induced DNA damage is to further augment activation of poly(ADP-ribose) polymerase 1 leading to ATP depletion during necrosis.

A host of inhibitors oppose these apoptosis pathways. They include Fas-associated via death domain-like interleukin-1β–converting enzyme inhibitory protein which inhibits DISC assembly and function, antiapoptotic Bcl-2 proteins that block release of mitochondrial apoptogens, and IAP family members that inhibit already activated downstream caspases as described. Although these inhibitors act on either the DR or mitochondrial apoptosis pathways, apoptosis repressor with caspase recruitment domain inhibits both pathways by disrupting DISC assembly and inhibiting Bax activation. Apoptosis repressor with caspase recruitment domain expression was initially believed to be limited to cardiac and skeletal myocytes and neurons, but recent data show that it is also induced at high levels in cancer cells and hypoxic pulmonary artery smooth muscle cells in vivo.

Mitochondrial Signaling: Necrosis

In contrast to OMM permeabilization in apoptosis, the defining event of necrosis at the mitochondria is opening of the mPTP, a pore in the inner mitochondrial membrane. In healthy mitochondria, the inner mitochondrial membrane is impermeable to water, ions, and even single protons. Because substrates are metabolized in the mitochondrial matrix resulting in the transport of electrons along the respiratory chain, protons are pumped from the matrix to the intermembrane space. This creates an electrochemical gradient (mitochondrial membrane potential) between the intermembrane space and matrix, which provides the potential energy necessary to drive ATP synthesis. Necrotic stimuli, such as Ca^{2+}, trigger opening of the mPTP. Ca^{2+}-induced mPTP opening can be potentiated by ROS, alkalosis, and depletion of ATP or ADP. Opening of the mPTP causes abrupt loss of mitochondrial membrane potential leading to cessation of mitochondrial ATP synthesis. In addition, mPTP opening allows water to rush down its osmotic gradient into the matrix, leading to mitochondrial swelling, and sometimes frank rupture of the OMM. Although rupture of the OMM can cause release of cytochrome c and activate caspases, it is unclear how much engagement of downstream apoptosis signaling contributes to cell death in the mitochondrial necrosis pathway given the other cataclysmic events precipitated by mPTP opening. However, as discussed below, potential caspase activation during necrosis complicates interpretation of assays such as terminal deoxynucleotidyl transferase dUTP nick-end labeling, which are traditionally assumed to be specific to apoptosis.

Despite extensive research in the field, the components of the mPTP remain unknown. The adenine nucleotide translocase and phosphate carrier in the inner mitochondrial membrane, voltage-dependent anion channel, and peripheral benzodiazepine receptor in the OMM, hexokinase which is loosely attached to the cytosolic face of the OMM, and cyclophilin D (a peptidyl-prolyl cis-trans isomerase) in the matrix have been proposed to be components of the pore. However, genetic studies have excluded adenine nucleotide translocase, voltage-dependent anion channel, and cyclophilin D as core pore components, although adenine nucleotide translocase and cyclophilin D are important positive regulators of pore opening.

Necrosis can occur as a primary event or secondary to apoptosis, the latter when the disposal of apoptotic bodies is delayed. Delayed clean-up occasionally occurs in vivo and is almost always observed at late time points in cell culture. In primary necrosis, mPTP opening occurs early, before cytochrome c release. If mPTP opening takes place during apoptosis, it occurs coincident with or after cytochrome c release. In this case, mPTP opening may result from caspase-dependent events. Although the kinetics differ markedly, these observations explain why loss of mitochondrial membrane potential may provide a marker for both necrosis and apoptosis.

How cell death stimuli connect with the mitochondrial necrosis machinery is incompletely understood. Some classic activators of this pathway, such as ischemia and ischemia-reperfusion (I/R), induce mPTP opening through Ca^{2+} and ROS. In addition, activators of the DR necrosis pathway may ultimately engage the mitochondrial necrosis pathway through links that were previously discussed. It is likely, however, that additional connections/pathways exist.

ER-Mediated Apoptosis and Necrosis

The ER mediates the synthesis and proper folding of multiple proteins, some posttranslational modifications, trafficking of newly synthesized proteins to the Golgi apparatus, lipid biosynthesis, and Ca^{2+} homeostasis. These effects are critical for normal cellular functioning. Under certain conditions, however, the ER can also mediate cell death, both apoptosis and necrosis. Considerable controversy exists as to the precise mechanisms by which the ER contributes to cell death, and the mechanisms that mediate the switch from adaptation to death. Although adaptive and death responses could be mediated by parallel pathways, the involvement of shared signaling components implicates the same pathways in both outcomes. For example, misfolded proteins in the ER lumen elicit a response

mediated by ER transmembrane sensors protein kinase R-like ER kinase, inositol-requiring protein 1α, and activating transcription factor 6. These proteins activate complex transcriptional and posttranscriptional cascades to reestablish ER homeostasis. However, it is thought that, when various ER stresses (eg, misfolded proteins, oxidative stress, certain lipids) fail to be resolved in a timely manner, death may result.

Although the precise ER-specific machinery by which cell death is promoted remains incompletely understood, the transcription factor C/EBP homologous protein has been clearly implicated. C/EBP homologous protein, which is activated downstream of the ER transmembrane sensors, induces the expression of proapoptotic proteins Bcl-2-interacting mediator, tetracycline response element-binding protein 3, and DR5, and represses that of Bcl-2. Another important death mediator is Ca^{2+}, which transits from the ER lumen to the mitochondria, to trigger apoptosis or necrosis through mechanisms that are discussed in the section on cross talk between mitochondrial apoptosis and necrosis pathways. Less clear are potential roles for various caspases, c-Jun N-terminal kinases, other ER membrane proteins, and cleavage of multiple mRNAs by inositol-requiring protein 1α, which also possesses endonuclease activity.

Autophagy-Associated Cell Death

Autophagy is a process in which the cell breaks down its own proteins and lipids. This provides energy during periods of starvation and stress, a means for the disposal of long-lived proteins, and a mechanism for protein quality control. Accordingly, in organisms ranging from yeast to mammals, autophagy is a survival mechanism. That said, too much autophagy has been hypothesized to cause cell death, a process referred to as autophagic cell death or, more accurately, as autophagy-associated cell death. It is plausible that self-cannibalization could result in cell death. However, at this point in time, a direct causal link between autophagy and cell death has not been definitively demonstrated. One impediment in establishing this connection is the absence of markers for autophagy-associated death in distinction to the existence of abundant markers for autophagy itself. In most experiments, an intervention is used to alter rates of autophagy, the success of which is confirmed with autophagy markers, and this manipulation is then correlated with histological markers of cell death (eg, terminal deoxynucleotidyl transferase dUTP nick-end labeling). Although it is possible that autophagy kills cells indirectly through another form of cell death, an autophagy-specific mode of killing has not been identified. Questions remain even regarding the interpretation of electron micrographs showing presumably dead or dying cells that contain

autophagic vacuoles because it is unclear whether autophagy in this situation represents a pathogenic mechanism, a compensatory process, or is unrelated to the presumed cell death. There are, however, some convincing data supporting a role for autophagy in cell death, eg, during regression of the salivary gland in *Drosophila* development. In addition, we highlight studies linking autophagy to cell death during myocardial infarction and heart failure in the section on heart disease below.

Although a dedicated machinery for autophagy-associated cell death has not been identified, physical and functional connections between key autophagy and cell death proteins have been recognized and might provide insights into interrelationships between these processes. Beclin-1, a protein involved in autophagosome formation, contains a BH3 domain analogous to those in BH3-only proteins, which as discussed above promote apoptosis. The Bcl-2-Beclin-1 interaction inhibits the proautophagic function of Beclin-1 in response to starvation without interfering with antiapoptotic function of Bcl-2. Moreover, multiple BH3-only proteins can displace Beclin-1 from Bcl-2 to promote autophagy.

Connections Between Cell Death Pathways

We have previously discussed connections that link (1) DR apoptosis with mitochondrial apoptosis pathways (eg, Bid) and (2) DR apoptosis with DR necrosis pathways (caspase-8 activity as a decision point in apoptosis versus necrosis in this pathway). In this section, we consider molecules/pathways connecting (1) necrosis signaling at DRs with that at the mitochondria and (2) mitochondrial apoptosis and necrosis pathways.

Cross Talk Between DR and Mitochondrial Necrosis Pathways

As previously discussed, activation of the DR pathway signals necrosis when caspase-8 is inhibited. First, induction of necrosis in this paradigm is abrogated by the absence of Bax/Bak or cyclophilin D, genetically linking DR and mitochondrial necrosis events. Second, RIP1 translocates to the mitochondria when activated in the DR necrosis pathway, although its mitochondrial actions are not yet understood. Third, activation of RIP1 and RIP3 in the DR pathway stimulates ROS production through nicotinamide adenine dinucleotide phosphate oxidase 1 and glutamate dehydrogenase 1/glutamate ammonia ligase/glycogen phosphorylase

1 activation respectively, and as discussed, ROS is a strong potentiator of Ca^{2+}-induced mPTP opening. Fourth, as discussed previously, RIP3 activation in the DR pathway also triggers cell death through phosphorylation of the mitochondrial phosphatase phosphoglycerate mutase 5. Other connections are likely to become evident as these pathways are understood in more detail.

Cross Talk Between Mitochondrial Apoptosis and Necrosis Pathways

We have previously discussed some connections between these pathways including how OMM rupture (not permeabilization) in necrosis may result in cytochrome c release, and how caspase activation in apoptosis may trigger late mPTP opening. Another important connection involves Bcl-2 proteins, which unite apoptosis and necrosis signaling at the mitochondria through their effects on Ca^{2+} handling at the ER. Bax, which induces OMM permeabilization during apoptosis, also increases the concentration of Ca^{2+} in the ER lumen, such that a larger Ca^{2+} bolus is released when the ER is presented with a death stimulus. ER Ca^{2+} transits to the mitochondria either through the cytoplasm or via direct connections between mitochondria and ER. Increases in mitochondrial Ca^{2+} can trigger mPTP opening and necrosis or apoptosis through mechanisms that have not yet been defined. Bcl-2 opposes these Bax-induced effects on both the mitochondria and ER.

X. CELL DEATH in HEART DISEASE

Myocardial Infarction

Surgical occlusion of the left coronary artery is used as a surrogate for acute thrombosis in animal models of ST-segment elevation myocardial infarction. This process is usually studied in the context of reperfusion (I/R) because of the clear benefit of restoring blood flow in human myocardial infarction. It should be noted, however, that despite the net effect of reperfusion to reduce infarct size, the introduction of blood into an ischemic zone generates ROS, Ca^{2+}, and alkalosis, all inducers of mPTP opening. For this reason, significant research is directed toward reducing reperfusion injury. Another point relevant to interpreting data from rodent models of I/R is that, despite rare reports to the contrary, it is unusual for genetic or pharmacological manipulations to reduce infarct size in the setting of prolonged

ischemia without reperfusion (permanent occlusion), another reason why most studies use I/R.

Cell Death in Myocardial Infarction

In both permanent occlusion and I/R models of myocardial infarction, a large burst of cell death takes place within the area rendered ischemic over the first 6 to 24 hours. Lesser amounts of cell death takes place in the periinfarct zone, initially the result of residual ischemia, but persisting due to cardiac remodeling driven by the loss of contractile units in the infarct. A yet lower magnitude of cell death continues for months in the remote myocardium as remodeling progresses. In this section, we focus on cardiac myocyte death in the ischemic zone.

During myocardial infarction, cardiac myocytes in the ischemic zone die by both apoptosis and necrosis. Surprisingly, the magnitudes of each form of cell death remain unclear. The impediment has been limitations of current assays to definitively distinguish between apoptosis and necrosis in tissue from animals subjected to myocardial infarction. For example, although the primary consequence of mPTP opening during necrosis is cessation of ATP synthesis, the accompanying mitochondrial swelling can result in OMM rupture and cytochrome c release. It is unclear how often OMM rupture occurs in this situation, but the potential release of cytochrome c confounds the interpretation of assays based on caspase activation and DNA fragmentation (eg, terminal deoxynucleotidyl transferase dUTP nick-end labeling). Solutions include the direct evaluation of plasma membrane integrity in vivo using a variety of approaches and electron microscopy, although the latter is limited by differential sensitivities for the detection of necrotic versus apoptotic cells. Although these techniques have been used to some extent, a rigorous quantification of apoptosis and necrosis during myocardial infarction is needed.

Apoptosis in Myocardial Infarction

Multiple studies have demonstrated a causal connection between cardiac myocyte apoptosis and myocardial infarction. Both the DR and mitochondrial pathways have been shown to be critical. Hearts of mice lacking Fas exhibit smaller infarcts in response to I/R, when studied as isolated preparations or in vivo. Given that death signals related to I/R potently activate the mitochondrial pathway, the

reasons underlying the importance of the DR pathway in this process are not obvious. One explanation may be that death ligands themselves are important mediators of I/R, and in support of this, Fas ligand appears in the coronary effluent of isolated hearts during the reperfusion phase. Another possibility may be that activation of the DR pathway provides another input into activation of the mitochondrial apoptosis pathway through truncated Bid.

Cardiac-specific overexpression of Bcl-2 decreases infarct size and cardiac dysfunction after I/R in vivo. In addition, deletion of Bax reduces infarct size in isolated hearts subjected to I/R. Bax deletion has also been reported to cause mild reductions in infarct size after permanent occlusion in vivo. Absence of p53 upregulated modulator of apoptosis, a p53 responsive BH3-only protein, reduces infarct size in isolated, perfused hearts subjected to I/R. Thus, Bcl-2 family members modulate infarct size.

Cardiac overexpression of cellular IAP 2 results also in smaller infarcts in isolated perfused hearts subjected to I/R. This effect may result from the inhibition of already activated downstream caspases by IAPs by cellular IAP 2 and its K63-polyubiquitination of RIP1 which activates the DR survival pathway. UCF-101, a small molecule inhibitor of the serine protease activity of Omi/high temperature requirement protein A2, decreases infarct size after I/R. Pan-caspase inhibitors provide varying degrees of reduction in the size of infarcts elicited by I/R. Overexpression of apoptosis repressor with caspase recruitment domain, which inhibits both DR and mitochondrial apoptosis pathways, also decreases infarct size after I/R. The fact that multiple manipulations of apoptosis pathways affect infarct size provides confidence that this form of cell death is involved in myocardial infarction.

Necrosis in Myocardial Infarction

Regulated necrosis has also been demonstrated to play a role in the development of myocardial infarction. Necrostatin, the inhibitor of the kinase activity of RIP1, reduces infarct size in response to I/R in vivo. Interestingly, its cardioprotective effect is dependent on the presence of cyclophilin D, suggesting connections between RIP1 and mitochondrial necrosis events.

Bax and Bak have recently been shown to regulate necrosis. In addition to reducing infarct size, deletion of Bax and Bak markedly reduces the degree of necrotic injury in the hearts of mice subjected to I/R. These effects occur through a pathway distinct from the regulation of apoptosis by Bax and Bak, as evidenced by

the ability of oligomerization deficient Bax mutants, which cannot support apoptosis, but retain the ability to mediate necrosis.

Mice lacking cyclophilin D, a positive regulator of mPTP opening, demonstrate decreased infarct size after I/R. Pharmacological inhibition of cyclophilin D, using cyclosporine A or sangliferin A, also reduces infarct size. A pilot study has translated this work to a small number of patients with ST-segment elevation myocardial infarction. When superimposed on angioplasty and stenting, cyclosporine A resulted in statistically significant reduction in infarct size as measured by serum levels of creatine kinase, but not troponin I, and by magnetic resonance imaging. Although significant reductions in infarct size persisted in 6 months postmyocardial infarction, only a nonstatistically significant trend toward preserved cardiac function was observed. Thus, further work is needed to assess the efficacy of this cardioprotective strategy in humans.

Taken together, these studies demonstrate that both apoptosis and necrosis contribute to the pathogenesis of myocardial infarction.

Autophagy-Associated Death in Myocardial Infarction

Autophagy is induced during both I/R and permanent occlusion. However, the mechanisms and the consequences of this induction appear to be different. During permanent occlusion, 5' AMP-activated protein kinase is activated, and inhibits mammalian target of rapamycin, a potent inhibitor of autophagy. Consequently, autophagy is induced. Inhibition of autophagy by transgenic overexpression of dominant negative 5' AMP-activated protein kinase resulted in worsening of infarct size in response to permanent occlusion. Similar results were obtained when autophagy was inhibited by overexpression of Rheb, overexpression of a dominant negative form of glycogen synthase kinase 3β, or deletion of 1 allele of glycogen synthase kinase 3β. Thus, consistent with the survival role of autophagy during starvation, these data suggest that autophagy protects the myocardium during prolonged ischemia. During I/R, however, Beclin-1 levels increase to activate autophagy. Mice, in which 1 allele of Beclin-1 has been inactivated, exhibit smaller infarcts in this situation. Similar results were found when autophagy was decreased by loss-of-function manipulations of glycogen synthase kinase 3β as described above. These and other studies suggest that autophagy is associated with a protective role during ischemia and a pathogenic role during I/R. Further

investigation is needed, however, to determine the extent to which alterations in autophagy explain these changes in infarct size.

XI. CELL DEATH and HEART FAILURE

Apoptosis in Heart Failure

In contrast to myocardial infarction in which there is an explosive and short-lived burst of cell death, the absolute percentage of apoptotic cardiac myocytes in failing human hearts is low (0.08%–0.25% as assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling). However, this percentage of cardiac myocyte apoptosis is ≈10- to 100-fold higher than that observed in control hearts (0.001%–0.01%). These data suggest the hypothesis that low, but elevated levels of cardiac myocyte apoptosis, result over time in cumulative loss of cardiac myocytes and heart failure. This possibility was first tested in transgenic mice with a conditionally activatable procaspase-8 allele, which showed that rates of cardiac myocyte apoptosis as low as 0.023% elicit a lethal dilated cardiomyopathy. Control mice overexpressing an enzymatically dead procaspase-8 remained normal. These data establish the sufficiency of clinically relevant levels of apoptosis to induce heart failure.

Conversely, the necessity of cardiac myocyte apoptosis for heart failure was tested using pan-caspase inhibition in a model of peripartum cardiomyopathy. This was induced by cardiac-specific overexpression of Gαq, a surrogate for humoral stimuli relevant to heart failure. Pregnancy precipitated lethal heart failure in 30% of Gαq transgenic mice. Pretreatment with a pan-caspase inhibitor reduced cardiac myocyte apoptosis, preserved heart function, and completely rescued mortality. These data demonstrate the necessity of cardiac myocyte apoptosis for heart failure in this model. These concepts have also been extended to other models. For example, after myocardial infarction, deletion of Bcl-2/adenovirus E1B 19kD-interacting protein 3, a BH3-like protein, reduced pathological remodeling in the periinfarct zone and resultant heart failure.

Necrosis in Heart Failure

Cardiac myocyte necrosis may also play a role in heart failure. Cardiac myocyte-specific transgenic overexpression of the β2-α subunit of the L-type Ca^{2+} channel resulted in Ca^{2+} overload, mPTP opening, necrosis, and cardiac dysfunction. This

phenotype was rescued by deletion of *peptidylprolyl isomerase F* encoding cyclophilin D, but not overexpression of Bcl-2, suggesting that heart failure in this model is attributable to cardiac myocyte necrosis. Similarly, doxorubicin-induced cardiomyopathy was ameliorated by knockout *peptidylprolyl isomerase F*. In contrast to myocardial infarction, involvement of necrosis in heart failure is somewhat unexpected. Although this interpretation may be correct, it is important to also consider recently discovered effects of cyclophilin D on cardiac metabolism. Future work is needed to determine the magnitude of cardiac myocyte necrosis in failing hearts and the general applicability to pathogenesis of this syndrome.

Autophagy-Associated Death in Heart Failure

A previous study of failing human hearts has suggested that autophagy-associated cell death is the most common form of cellular demise during heart failure. However, the markers used to diagnose various forms of cell death in the present study were not specific. Stronger data concerning the relationship of autophagy and heart failure have been provided by genetic loss- and gain-of-function studies. Autophagy protein 5 deletion in the heart precipitates ventricular enlargement and cardiac dysfunction after hemodynamic overload implying that autophagy is a compensatory mechanism during heart failure. In contrast, Beclin-$1^{+/-}$ mice subjected to pressure overload exhibited decreased pathological remodeling and cardiac dysfunction whereas Beclin-1 overexpression resulted in the opposite. The explanation for the conflicting results in the autophagy protein 5 and Beclin-1 studies is not known, but may be related to differences in the genetic manipulations or apparent severity of pressure overload. Therefore, the role of autophagy in the pathogenesis of pressure overload-induced heart failure is not clear. On the other hand, deletion of 1 allele of Beclin-1 worsens cardiac remodeling and function and mortality in response to proteotoxic stress induced by transgenic overexpression of the R120G mutant of αβ-crystallin, a model of desmin-related cardiomyopathy. Thus, in keeping with its role in disposing of defective proteins, autophagy plays a protective role in heart failure initiated by proteotoxicity. Taken together, these data highlight that autophagy may be protective in response to some cardiomyopathic stimuli and pathogenic in response to others.

Concluding Remarks

The present review discusses the role of cell death in the major syndromes that affect the heart: myocardial infarction and heart failure. Although myocardial infarction and heart failure are complex and involve multiple cellular processes, the data indicate that cell death plays a critical role in the pathogenesis of both syndromes. The regulated nature of much of the cell death in these diseases opens up the possibility of manipulating death pathways to therapeutic advantage. Given its acute nature, myocardial infarction is the most attractive target. An important issue in this setting is how the drug will access tissue in which the blood supply is compromised. One possibility is drug delivery at the time of reperfusion. However, administration even before reperfusion may have beneficial effects on the periinfarct region as well as potentially extending the window for effective reperfusion. Heart failure may also be a viable target, but potential oncogenic effects of chronic cell death inhibition are a concern. To circumvent this obstacle requires the development of approaches to target drug to the myocardium. The hope is that, in combination with therapies directed at atherosclerosis and plaque rupture, small molecules approaches to decrease the susceptibility of the myocardium to cell death will limit tissue damage and ultimately reduce mortality.

XII. CLINICAL PATHOPHYSIOLOGY of HYPOXIC ISCHEMIC BRAIN INJURY AFTER CARDIAC ARREST: A “TWO-HIT” MODEL

Hypoxic ischemic brain injury (HIBI) after cardiac arrest (CA) is a leading cause of mortality and long-term neurologic disability in survivors. The pathophysiology of HIBI encompasses a heterogeneous cascade that culminates in secondary brain injury and neuronal cell death. This begins with primary injury to the brain caused by the immediate cessation of cerebral blood flow following CA. Thereafter, the secondary injury of HIBI takes place in the hours and days following the initial CA and reperfusion. Among factors that may be implicated in this secondary injury include reperfusion injury, microcirculatory dysfunction, impaired cerebral autoregulation, hypoxemia, hyperoxia, hyperthermia, fluctuations in arterial carbon dioxide, and concomitant anemia.

Clarifying the underlying pathophysiology of HIBI is imperative and has been the focus of considerable research to identify therapeutic targets. Most notably, targeted temperature management has been studied rigorously in preventing

secondary injury after HIBI and is associated with improved outcome compared with hyperthermia. Recent advances point to important roles of anemia, carbon dioxide perturbations, hypoxemia, hyperoxia, and cerebral edema as contributing to secondary injury after HIBI and adverse outcomes. Furthermore, breakthroughs in the individualization of perfusion targets for patients with HIBI using cerebral autoregulation monitoring represent an attractive area of future work with therapeutic implications.

We herein provide an in-depth review of the pathophysiology of HIBI to critically evaluate current approaches for the early treatment of HIBI secondary to CA. Potential therapeutic targets and future research directions are summarized

Background

Cardiac arrest (CA) is a major cause of mortality and neurologic disability. The incidence of out-of-hospital CA is approximately 80 patients per 100,000 persons annually. Despite advances in resuscitation, outcomes remain dismal, with 10% of patients surviving until hospital discharge and 5% experiencing full neurologic recovery.

The primary determinant of outcome after CA is hypoxic ischemic brain injury (HIBI). HIBI is the primary cause of death in 68% of inpatient CA and in 23% of out-of-hospital CA. HIBI is associated with significant neurologic disability, ranging from mild cognitive deficits to minimally conscious and persistent vegetative states. Consequently, considerable effects on quality of life and incidence of psychiatric comorbidities, such as depression, anxiety, and posttraumatic stress disorder, are highly prevalent in HIBI survivors. The vast spectrum of acute and chronic HIBI phenotypes requires detailed understanding of cerebral physiologic perturbations that occur after CA and make clarifying the pathophysiology essential.

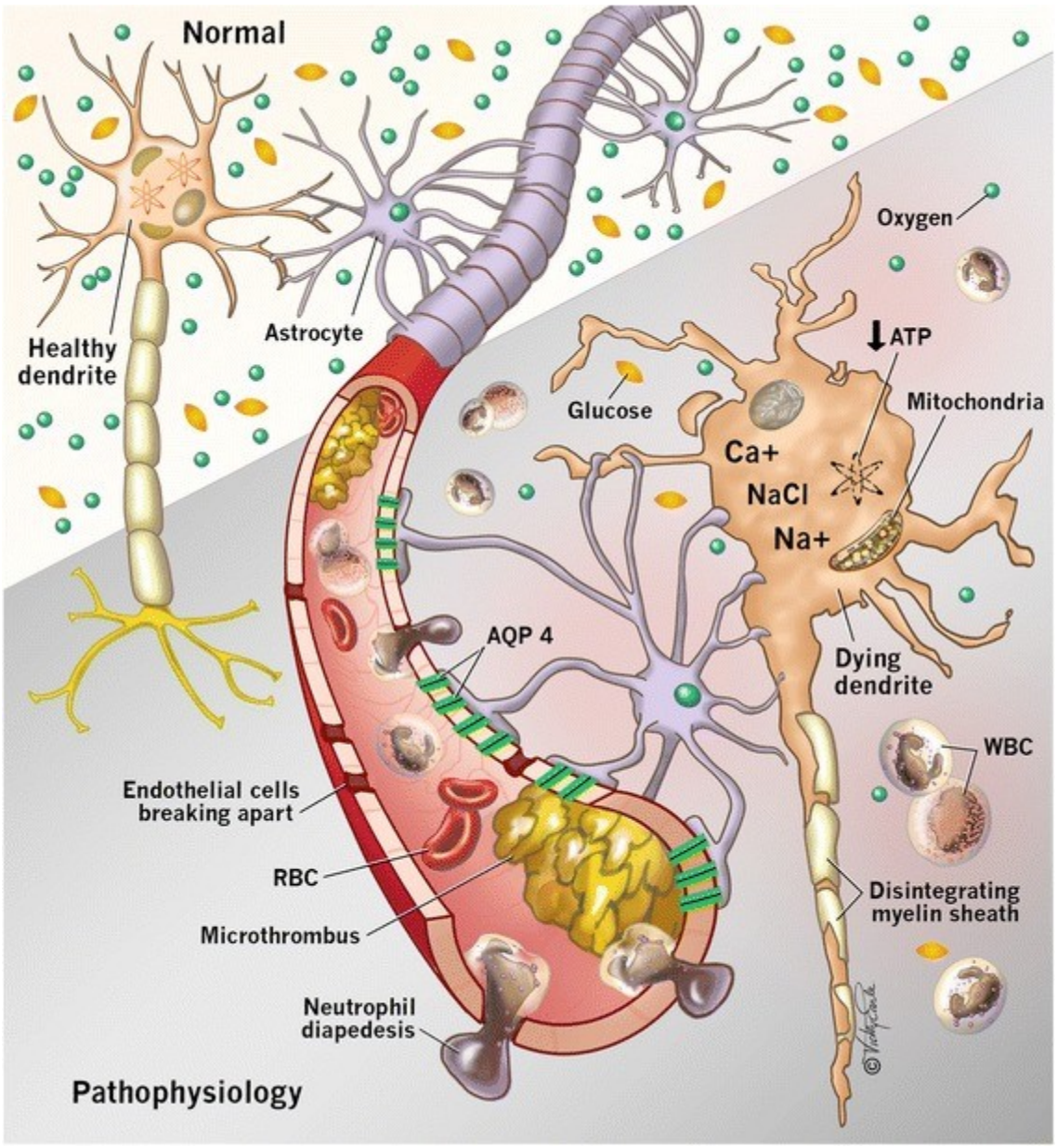
Management of HIBI is focused on limiting secondary injury by optimizing the balance between cerebral oxygen delivery (CDO_2) and use. Despite rigorous research, HIBI outcomes have not appreciably changed over 20 years. This stagnation is in contrast with improved outcomes in other critical care diseases.

Considerable opportunities remain to delineate the pathophysiology of HIBI. HIBI pathophysiology is a “two-hit” model, being determined by primary injury from immediate cessation of CDO_2 during CA and secondary injury occurring after resuscitation. We present a narrative review of a two-hit model of HIBI pathophysiology as it pertains to physiologic parameters involved in maintaining

the balance of CDO_2 and use. We highlight advances pertaining to cerebral autoregulation, optimal hemoglobin, carbon dioxide, cerebral edema, normobaric hyperoxia, and targeted temperature management.

Primary injury

During CA, cessation of CDO_2 occurs with resultant neuron ischemia and cell death within minutes (Fig. 1). The cerebrum consumes 20% to 25% of cardiac output to maintain function. The brain is devoid of nutrient stores, and consequently neuroglycopenia and metabolic crisis occur within minutes after CA, leading to cell death.



A schematic demonstrating the various microvascular and cellular pathophysiologic consequences which occur during the primary and secondary injury in hypoxic ischemic brain injury (HIBI). Decreased cerebral oxygen delivery manifests as reduced neuronal aerobic metabolism, causing reduced cellular adenosine triphosphate (ATP) production. Intracellular calcium accumulation leads to mitochondrial toxicity and further reduced ATP production. Inability to sustain cellular respiration results in cell death and apoptosis. Additionally, in the microvasculature, endothelial dysfunction leads to a porous blood-brain barrier, formation of cerebral edema, formation of microthrombi and limitation of cerebral blood flow with exacerbation of cellular ischemia. *AQP 4* Aquaporin-4, *RBC* Red blood cells, *WBC* White blood cells

As CDO_2 decreases, adenosine triphosphate production halts, causing cessation of energy-dependent ion channel function. Subsequent intracellular Na^+ accumulation results in cytotoxic edema. Depletion of adenosine triphosphate leads to anaerobic metabolism, cerebral lactate accumulation, and intracellular acidosis. Additionally, cellular ischemia causes intracellular Ca^{2+} influx through *N*-methyl-D-aspartate channels, which activates lytic enzymes and mitochondrial dysfunction, thereby depleting adenosine triphosphate further. Finally, excitatory neurotransmitter release activates lipases and proteases, which leads to apoptosis.

Clinically, loss of neurologic function is manifested by a decreased level of consciousness after global cerebral ischemia. Historically, Rossen et al. demonstrated that cessation of cerebral blood flow (CBF) by neck cuff insufflation to 600 mmHg in humans precipitated acute decreased level of consciousness within 10 seconds. Decreased level of consciousness after CA occurs within 20 seconds after onset of ventricular fibrillation. Loss of neurologic function has been demonstrated by isoelectric electroencephalography in observational studies. Pana et al. identified human studies demonstrating isoelectric electroencephalography rhythms within 15 seconds and 30 seconds of asystole and ventricular fibrillation, respectively. These findings are corroborated by animal studies establishing a similar timeline of 10 to 30 seconds from the onset of cerebral ischemia to isoelectric electroencephalography.

Although primary injury causes substantial neuronal loss, the ensuing post resuscitation additive cerebral injury accounts for significant cerebral ischemia and cellular death. The key pathophysiologic factors that are implicated in secondary injury are physiologic modifiers involved in maintaining the balance between CDO_2 and use. We next discuss secondary injury and physiologic determinants that are targets of therapeutic interventions after HIBI.

Secondary injury

Secondary injury is the additive cerebral injury characterized by an imbalance in post resuscitation CDO_2 and use, ultimately culminating in neuronal death. It begins immediately after return of spontaneous circulation (ROSC). Structures especially susceptible include the hippocampi, thalami, cerebral cortex, corpus striatum, and cerebellar vermi, owing to highly metabolically active tissue. Aside from hypothermia, there are limited studies examining physiologic variables that exacerbate secondary injury. Table 1 summarizes the mechanisms of secondary injury.

Table 1

Summary of mechanisms of secondary brain injury after hypoxic ischemic brain injury.

Pathophysiology	Mechanisms	Consequences
Microvascular dysfunction	Microthrombi, cerebral vasoconstriction, blood-brain barrier disruption	Increased cerebrovascular resistance, decreased CBF, decreased cerebral O_2 delivery, vasogenic cerebral edema
Cerebral edema	Vasogenic cerebral edema, cytotoxic cerebral edema	Increased ICP and decreased CPP, decreased CBF, herniation, brain death
Anemia	Decreased arterial oxygen content	Cerebral ischemia
Impaired autoregulation	Narrowed and right-shifted autoregulation	Pressure passive cerebral hemodynamics, cerebral ischemia and hyperemia
Carbon dioxide	Hypocapnia-induced vasoconstriction, hypercapnia-induced vasodilation	Decreased CBF, cerebral ischemia, increased ICP, decreased CPP, decreased CBF
Hyperoxia	Increased O_2 free radicals	Neuronal cell dysfunction and cell death

Microcirculation and reperfusion injury

After ROSC, microcirculatory perturbations lead to further neuron dysfunction. The cerebrovascular endothelium plays a critical role in maintaining blood-brain barrier integrity, regulation of microcirculatory blood flow, and release of auto anticoagulant mediators. Endothelial functions are compromised, and biomarkers of cerebrovascular endothelial injury are associated with adverse outcomes in HIBI.

Following ROSC, reperfusion injury causes neuronal dysfunction despite restoration of CDO_2. An initial period of cerebral hyperemia is followed by hypoperfusion, resulting in a "no-reflow" state that exacerbates secondary injury. Mechanisms implicated in the no-reflow state include impaired vasomotor regulation, decreased nitric oxide production, and resultant vasoconstriction. Extravasation of intravascular water through a porous blood-brain barrier with perivascular edema leads to increased intravascular viscosity and cerebrovascular resistance. Other mechanisms implicated in reperfusion injury include free radical release, glutamate production, and intracellular Ca^{2+} accumulation.

Endothelial auto anticoagulant dysfunction causes diffuse microthrombi in the cerebrovasculature. Concomitant impaired vasodilation causes increased cerebrovascular resistance and reduces CBF. Interventional studies demonstrate that heparin and tissue plasminogen activator improve microcirculatory flow. These findings have not translated into improved outcomes when evaluated prospectively, however. Finally, intravenous prostacyclin is suggested to promote endothelial function through vasodilatory and antiplatelet effects, but clinical studies are not yet available. Table 2 summarizes mechanisms involved in reperfusion injury.

Table 2

Pathophysiologic summary of cerebral reperfusion injury after cardiac arrest

Pathophysiology	Mechanisms	Consequences
Endothelial dysfunction	Impaired vasomotor control of blood flow, microthrombi formation, blood-brain barrier disruption	Impaired blood flow in microcirculation and limited oxygen delivery, cerebral edema

Pathophysiology	Mechanisms	Consequences
Free radical formation	Activation of lytic cellular enzymes	Neuronal apoptosis and cell death
Intracellular Ca^{2+} accumulation,	Mitochondrial toxicity, activation of cellular lytic enzymes	Reduced adenosine triphosphate production, cell death, apoptosis
Impaired nitric oxide,	Vasoconstriction, "no reflow"	Reduced cerebral blood flow, cerebral ischemia
Excitatory neurotransmitter release	Glutamate release	Excitotoxicity, seizures, apoptosis, cell death

Hemoglobin

Hemoglobin is a major determinant of arterial oxygen content. In animal studies of traumatic brain injury, concomitant anemia exacerbates secondary injury from apoptosis. However, physiologic benefits of improved CDO_2 from transfusion must be balanced by risks associated with exogenous red blood cells. Although hemoglobin <70 g/L is the accepted transfusion threshold for nonbleeding critical care patients, it remains unclear if a liberal threshold is appropriate for patients with brain injury, who are susceptible to secondary injury from anemia.

Evidence of anemia in contributing to secondary injury in HIBI is limited to observational studies. Nakao et al. conducted a retrospective study of 137 subjects with witnessed CA and established that higher admission hemoglobin was an independent predictor of a 28-day favorable neurologic outcome (OR 1.26, 95% CI 1.00–1.58). These findings were corroborated by Wang et al., who demonstrated an association with adverse outcome and lower admission hemoglobin. Recently, Johnson et al. conducted a multicenter observational study of 598 patients and found that favorable outcome patients had significantly higher hemoglobin (126 g/L versus 106 g/L, $p < 0.001$), a finding that persisted after adjustment.

Despite regression adjustment, admission anemia may be subject to strong residual or unmeasured confounding. It is unclear if admission hemoglobin captures the magnitude of effect that anemia has on secondary injury. Wormsbecker et al. accounted for this by investigating the relationship between mean hemoglobin over 7 days and neurologic outcome. They established that patients with a favorable outcome had significantly higher 7-day mean hemoglobin (115 g/L versus 107 g/L, $p = 0.05$). Furthermore, multivariable regression demonstrated that lower

7-day mean hemoglobin was associated with adverse outcome (OR 0.75 per 10 g/L change in hemoglobin, 95% CI 0.57–0.97). Importantly, Ameloot et al. established

a link between hemoglobin and a measure of brain oxygenation in an observational study of 82 patients. They found a linear association between hemoglobin and brain regional saturation of oxygen (rSO_2) using near-infrared spectroscopy, with hemoglobin <100 g/L being identified as a cutoff for lower rSO_2. Additionally, they demonstrated that mean hemoglobin concentration <123 g/L was associated with worse neurologic outcome, particularly in patients with $rSO_2 < 62.5\%$ (OR 2.88, 95% CI 1.02–8.16). Further research is required to establish an association between anemia with simultaneous brain hypoxia and investigate the effect of transfusion thresholds on outcome in HIBI.

Carbon dioxide

Partial pressure of arterial carbon dioxide ($PaCO_2$) modulates cerebrovascular resistance and CBF via its effects on vascular smooth muscle. Specifically, hypocapnia ($PaCO_2 < 35$ mmHg) induces cerebrovascular vasoconstriction and decreases CBF by about 2% to 3% for every 1 mmHg of $PaCO_2$. Clinically, hypocapnia reduces intracranial pressure (ICP) by reducing cerebrovascular volume. However, sustained hypocapnia can decrease CBF, increase cerebral oxygen extraction, and induce ischemia. Conversely, hypercapnia ($PaCO_2 > 45$ mmHg) is a cerebrovascular vasodilator that causes hyperemia, exacerbates ICP, and reduces CBF. Hypercapnia is also associated with excitotoxicity and increased cerebral oxygen demand. Importantly, $PaCO_2$ vascular reactivity is preserved after HIBI, making regulation of $PaCO_2$ clinically significant and a crucial determinant of CDO_2. The optimal $PaCO_2$ in individual patients is not known but presents a unique opportunity for advanced neurophysiologic monitoring using transcranial Doppler ultrasonography to evaluate CBF, ICP, and cerebrovascular resistance with varying $PaCO_2$ levels in HIBI.

Perturbations in $PaCO_2$ in HIBI have been evaluated in observational studies of HIBI. Roberts et al. conducted a retrospective study of 193 patients and investigated the effects of hypocapnia and hypercapnia compared with normocapnia ($PaCO_2$ 35–45 mmHg) on outcome. They demonstrated a relationship between adverse neurologic outcome and both hypocapnia (OR 2.43, 95% CI 1.04–5.65) and hypercapnia (OR 2.20, 95% CI 1.03–4.71). Exposure of hypocapnia and hypercapnia occurred 36% and 42% of the time after CA, respectively, making the exposure of CO_2 fluctuation significant. The authors followed that study with an analysis of a prospective registry of patients with HIBI

and found a significant association between normocapnia and good neurologic outcome (OR 4.44, 95% CI 1.33–14.85). Schneider et al. conducted a large multicenter database study of 16,542 patients with HIBI and investigated the effects of hypocapnia in HIBI, and they demonstrated a significant association between hospital mortality and hypocapnia (OR 1.12, 95% CI 1.00–1.24) compared with normocapnia. Given the sound biological plausibility and available clinical data, regulation of $PaCO_2$ warrants further systematic study to determine the precise optimal therapeutic strategy after HIBI. Critical links with intracranial physiologic parameters pertaining to ICP, CBF, and brain oxygenation and fluctuations in $PaCO_2$ are logical future goals in this field.

Cerebral edema

After HIBI, cerebral edema is a recognized complication that causes secondary injury. Because of a fixed overall intracranial volume, an increase in the parenchymal bulk from cerebral edema in HIBI can cause intracranial hypertension with resultant decreases in cerebral perfusion pressure, CBF, and CDO_2. This vicious cycle of cerebral edema precipitating increased ICP causes transtentorial herniation and brain death.

The origin of cerebral edema occurs as a result of either vasogenic or cytotoxic mechanisms. In the early stages, vasogenic edema emanates from fluid shifts from the intravascular to the cerebral interstitial space. Key to this process, aquaporin-4 is a membrane protein that transports water across cell membranes in the central nervous system. Aquaporin-4 proteins are located in perivascular astrocytic endfeet, processes, and ependyma. The aquaporin-4 perivascular pool is identified as the predominant cluster involved in the pathophysiology of cerebral edema after HIBI, with increased aquaporin-4 expression occurring within 48 h after the onset of cerebral ischemia. Interestingly, Nakayama et al. showed that 7.5% hypertonic saline attenuated cerebral edema in a wild-type mouse model of HIBI but had no effect in an aquaporin-4-knockout model, thereby demonstrating the importance of aquaporin-4 in the pathophysiology of cerebral edema and highlighting its therapeutic potential. Hypertonic saline administration also restores blood-brain barrier integrity mediated by aquaporin-4 in the hippocampi, cerebellum, cortex, and basal ganglia. Furthermore, Nakayama et al. established that achieving serum osmolality >350 mOsm/L with continuous infusion of conivaptan, a V_1 and V_2 antagonist, attenuated cerebral edema, thereby demonstrating that the effect of aquaporin-4 to decrease cerebral edema occurs through osmotic gradients, as opposed to a specific intravenous osmotic agent itself (e.g., 7.5% hypertonic saline).

Alternatively, cytotoxic edema originates from cellular metabolic crisis and intracellular energy depletion. Decreased adenosine triphosphate (Fig. 1) leads to energy-dependent ion channel failure and intracellular sodium and water retention. Rungta et al. established that the Na^+Cl^- receptor SLC26A11 is a critical modulator of intracellular transport of chloride and subsequent cerebral edema after ischemia. The authors showed that blockade of this receptor attenuated cytotoxic cerebral edema after HIBI. The role of Na^+Cl^- receptor antagonism after HIBI is yet to be clarified but represents a future therapeutic target.

Furthermore, sulfonylurea receptors are also implicated in the pathophysiology of cerebral edema after ischemia. Glyburide, a sulfonylurea receptor inhibitor, attenuates malignant cerebral edema after acute middle cerebral infarction. These findings are corroborated by animal studies that demonstrate sulfonylurea receptor antagonism decreases cerebral edema after neuronal ischemia.

Cerebral autoregulation

The brain has an innate ability to regulate blood flow to match metabolic demands. This phenomenon, termed *cerebral autoregulation*, allows the cerebrovasculature to undergo vasoconstriction and vasodilation over a range of mean arterial pressure (MAP) to maintain stable CBF. Cerebral autoregulation mitigates the effects of hypoperfusion (ischemia) and hyperperfusion.

The identification of individualized MAP targets after HIBI using cerebral autoregulation monitoring is an attractive concept that has garnered significant interest. Initially, Nishizawa et al. demonstrated a linear relationship between MAP and CBF (as indexed by jugular venous oximetry), suggesting complete dysfunctional cerebral autoregulation after HIBI. Thereafter, Sundgreen et al. constructed cerebral autoregulation curves for patients with HIBI by performing stepwise increases in MAP with norepinephrine and simultaneously estimating CBF with middle cerebral artery velocity on the basis of transcranial Doppler ultrasonography. Of the 18 patients studied by Sundgreen et al., cerebral autoregulation was absent in 8 and present in 10 patients. In five of ten patients with preserved cerebral autoregulation, the lower limit of autoregulation was right-shifted with a median MAP 114 mmHg (range 80–120 mmHg). This sentinel study demonstrated the heterogeneous nature of cerebral autoregulation in patients with HIBI and suggested that the lower limit of autoregulation may be significantly higher than traditional MAP targets after HIBI.

Recently, monitoring with near-infrared spectroscopy has garnered significant interest as a noninvasive method of optimal MAP identification and assessment of cerebral autoregulation after HIBI. Near-infrared spectroscopy measures the rSO_2 in the outermost 2 cm of the frontal lobe, represents the state of oxygenated hemoglobin in the microvasculature, and approximates CBF. Therefore, continually integrating fluctuations between MAP and rSO_2, a Pearson's product-moment correlation coefficient is generated. This correlation coefficient (COx) varies between −1 and +1. Positive COx values, where there is a positive and linear correlation between MAP and rSO_2, indicate dysfunctional autoregulation. Near-zero and negative COx values indicate intact autoregulation (i.e., rSO_2 remains relatively constant despite varying MAP).

The optimal MAP is identified as the MAP with the lowest value of COx, as shown in Fig. 3. Lee et al. demonstrated that COx identified the lower limit of autoregulation in a swine model of pediatric HIBI. Recently, Ameloot et al. retrospectively calculated COx using MAP and rSO_2 to indicate that autoregulation was intact in 33 of 51 subjects with HIBI. Thereafter, Pham et al. showed that COx was significantly higher in nonsurvivors of HIBI than in survivors. Although higher COx was associated with nonsurvivors, there was no association between rSO_2 and mortality. Recently, our research team demonstrated feasibility of monitoring COx in real time and identification of optimal MAP prospectively in 20 patients after CA. Subjects spent approximately 50% of time outside a ±5 mmHg range from the optimal MAP, and, importantly, the optimal MAP was consistently identified in 19 of 20 subjects. The concept of individualized perfusion pressures is emerging as an attractive therapeutic target and improved clinical outcome is associated if actual MAP is maintained within proximity of the identified optimal MAP.

It is imperative to recognize the downsides of targeting significantly right-shifted optimal MAP, particularly in patients with compromised left ventricular function after CA. Increasing afterload on a decompensated left ventricle can dramatically reduce stroke volume and cardiac output, placing the injured brain at increased risk of ischemia. Therefore, increased MAP targets in HIBI should be weighed against concurrent myocardial function. Considerable work remains to further delineate if individualized perfusion targets decrease brain hypoxia and secondary injury and are associated with improved neurologic outcome.

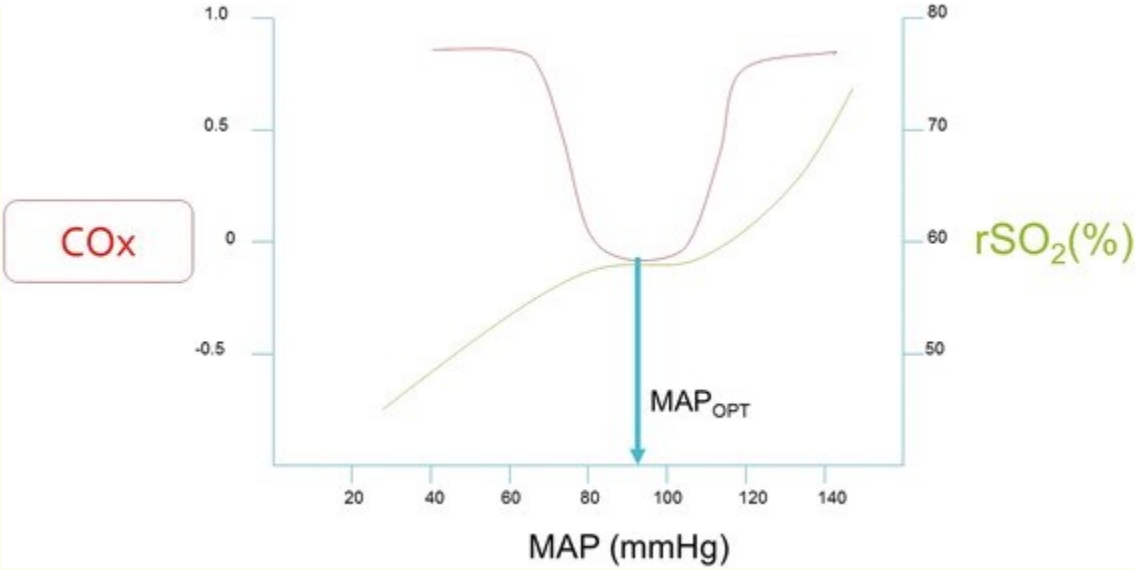


Fig. 3
The zone of preserved autoregulation after hypoxic ischemic brain injury appears to be narrowed and right shifted after cardiac arrest. Within the zone of autoregulation, regional saturation of oxygen (rSO_2) is stable owing to the innate vasoconstriction and vasodilation of the cerebral vasculature to maintain stable cerebral blood flow. Outside the zone of autoregulation, a linear relationship exists between rSO_2 and mean arterial pressure (MAP). By continually integrating the fluctuations of MAP and rSO_2 with one another, a correlation coefficient (COx) can be generated. The COx approaches negative values or near-zero within the preserved zone of autoregulation, resulting in a U-shaped curve. The nadir of the U-shaped curve represents the optimal MAP (MAP_{OPT}) for each individual patient

Temperature

Targeted temperature management has historically been the focus of considerable HIBI research. It is a mainstay in the management of HIBI by mitigating secondary injury after CA. At the cellular level, the beneficial effects of hypothermia are well documented. Cerebral metabolism is reduced by 5% to 10% per 1 °C decrease in core body temperature. In addition, global carbon dioxide production and oxygen consumption are decreased proportionally to reductions in core body temperature. By decreasing cerebral metabolism, hypothermia avoids excessive intracellular anaerobic metabolism, which leads to increased lactate production. Hypothermia also improves cerebral glucose use and allows available cellular energy stores to be used for necessary cellular functions in keeping with neuronal survival. Additional benefits of hypothermia include prevention of apoptosis by decreasing proapoptotic mediators such as p53, tumor necrosis factor α, and caspase enzymes while increasing expression of antiapoptotic proteins such as Bcl-2. Hypothermia

also prevents mitochondrial dysfunction, a key pathway involved in the promotion of apoptosis by release of cytochrome c oxidase into the cellular cytoplasm. Finally, hypothermia decreases inflammatory mediators such as the interleukin-1 family of cytokines as well as chemotaxis of leukocytes into cerebral interstitial tissue, reduces excitotoxic neurotransmitter release (glutamate and glycine), and decreases free radical production after HIBI. Sustained hypothermia also has detrimental physiologic effects pertaining to immune suppression, hemoconcentration, coagulopathy, arrhythmias, electrolyte disturbances, and hemodynamic instability, which must be weighed against the possible benefits. Furthermore, unintentional hypothermia can occur after CA, indicating possible severe damage to the key centers of thermoregulation, including the hypothalamus.

Hyperthermia is associated with numerous pathophysiologic sequelae that are potentially harmful after HIBI. Specifically, hyperthermia may increase blood-brain barrier permeability, leading to worsening cerebral edema, ICP,, and cerebral ischemia. Furthermore, hyperthermia increases glutamate production, which in turn causes intracellular Ca^{2+} influx, leading to neuronal cell death, seizures, and further secondary injury. Increased cerebral metabolism, hyperemic blood flow, and increased ICP are additional downstream consequences of uncontrolled hyperthermia in HIBI. Recently, it has been shown that hyperthermia is associated with dysfunctional autoregulation in patients with HIBI.

Clinical studies have established a firm link between hypothermia and improved outcome after CA. In 2002, two randomized controlled trials demonstrated marked improvement in clinical outcomes in patients with CA after ventricular fibrillation or ventricular tachycardia who were treated with hypothermia compared with standard of care. A persistent criticism of both studies was that the standard-of-care groups-maintained core body temperatures >37 °C, thereby exposing patients to the harmful effects of hyperthermia. This prompted a third recent randomized controlled trial comparing core body temperature control of 36 °C (normothermia) versus 33 °C (hypothermia) after CA. This pragmatic trial included patients with HIBI with all initial cardiac rhythms and ultimately did not demonstrate an appreciable benefit of hypothermia versus normothermia. Importantly, it must be stated that the maintenance of normothermia at 36 °C after CA requires active cooling. The negative effects of sustained hyperthermia and adverse outcomes after CA are well established, thereby reinforcing the importance of aggressive core body temperature control in patients following CA. It is possible that individualized temperature targets exist within patients with HIBI, and the inability of current studies to concurrently monitor cerebral metabolism, ICP, and biomarkers of neuron degeneration has limited our ability to make these patient-specific distinctions.

Normobaric hyperoxia

The dissolved portion of oxygen in plasma is a minor contributor to overall oxygen content. However, in disease states, this portion may have a pivotal role in ensuring adequate hemoglobin saturation for CDO_2 and overcome diffusion barriers to restore normal cellular metabolism. Augmenting arterial oxygen content is touted as a crucial modifiable factor in optimizing CDO_2 after HIBI, with normobaric hyperoxia being suggested to achieve this goal.

Upon ROSC, reperfusion injury occurs as a result of oxygen free radical production, which leads to intracellular oxidation. Examples include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl anion (OH^-), and nitrite (NO_2^-). Endogenous antioxidants balance the generation of free radicals and stabilize cellular function. Inadvertent normobaric hyperoxia in HIBI may tip this balance in favor of free radical production, cellular oxidation, and neuronal death. Although a systematic review of animal studies of HIBI suggested that increased neuron dysfunction occurs after normobaric hyperoxia, there was significant between-study heterogeneity with respect to ventilation strategies, timing and dose of normobaric hyperoxia, concomitant use of hypothermia, and the chosen primary outcomes. There are also several reported adverse effects associated with normobaric hyperoxia, including increased vascular resistance (cerebral, myocardial, and systemic), decreased CBF, seizures, and increased release neuronal degeneration biomarkers such as neuron-specific enolase.

Researchers in several studies have evaluated normobaric hyperoxia in HIBI, with conflicting results. Kuisma et al. conducted a randomized study of patients who were given 21% or 100% inspired oxygen after ROSC. The group that received 21% inspired oxygen exhibited lower serum levels of neuron-specific enolase than the normobaric hyperoxia group that did not undergo concomitant hypothermia. Kilgannon et al. interrogated the Project IMPACT database with more than 400,000 patients. They included patients with nontraumatic CA and cardiopulmonary resuscitation within 24 h prior to intensive care admission. Their objective was to examine the association between hyperoxia and mortality. Compared with the subjects in the normoxia group, subjects with normobaric hyperoxia (partial pressure of arterial oxygen [PaO_2] >300 mmHg) had higher associated in-hospital mortality (OR 1.8, 95% CI 1.5–2.2). Compared with normoxia, hypoxia ($PaO_2 < 60$ mmHg) was also associated with increased in-hospital mortality (OR 1.3, 95% CI 1.1–1.5). Spindelboeck et al. studied normobaric hyperoxia and hypoxemia during CA and found that both were associated with increased mortality, suggesting that the deleterious effects of normobaric hyperoxia may occur in early stages of HIBI. Finally, Bellomo et al.

conducted a retrospective analysis of patients with CA and demonstrated that normobaric hyperoxia and hypoxemia were associated with increased mortality; however, after adjustment, this relationship was no longer significant. Importantly, significant limitations in methodology should be noted, particularly the retrospective nature of these studies, the limitation of using mortality as a primary outcome in a brain injury population, and the fact that the definition of normobaric hyperoxia with a single $PaO_2 > 300$ mmHg does not capture the true biological exposure of patients to normobaric hyperoxia after CA. Furthermore, hypothermia was not routinely used in the aforementioned studies.

Additional retrospective analyses investigating the use of normobaric hyperoxia with concomitant hypothermia have addressed this shortcoming. Janz et al. demonstrated an association between adverse neurologic outcome and normobaric hyperoxia administration. These results are contrasted by those reported by Ihle et al. and Lee et al., who failed to show an association between normobaric hyperoxia and adverse neurologic outcome with concomitant hypothermia. Thereafter, a prospective study revealed an association between favorable neurologic outcome and higher mean PaO_2. Thus, concomitant hypothermia may play a role in modifying the deleterious effects of normobaric hyperoxia in HIBI.

Conclusions

HIBI pathophysiology is complex, with a significant contribution attributable to secondary injury. Researchers have investigated the effects of interventions aimed at preventing secondary injury, most notably hypothermia. Future targets of research include individualized perfusion targets, normobaric hyperoxia, transfusion triggers, and $PaCO_2$ goals.

XIII. NEBULIZATION PROCESS

To better understand the aerosolization therapies we must first describe the process in which medicines may be converted from solid or liquid to an aerosolized gaseous state. This conversion and therapeutics delivery method provides the basis of the BioZone System's Bio-Atmosphere interventional countermeasure potential to combat hyperinflammatory responses to infections, autoimmune responses, or radiation injury.

Nebulizer Medications and Their Benefits

Plausible treatment for SARS-CoV-2 infections have been found in nebulized treatments. The direct action of the medicinal aerosol to infected lung tissues is known to be important for opening airways and promoting better breathing. Nebulizers create this aerosol via nebulization which is inhaled by the patient. Unfortunately, the available nebulized treatments have not successfully addressed using the vast surface area of the pulmonary/capillary interface as a therapeutical delivery system to intervene in the cellular activation and manifestations thereof. The BioZone System's Bio-Atmosphere intends to deliver preventative measures to prevent cellular activation, block the mechanisms of activation, and provide therapeutics to deactivate the destructive and harmful cascading sequences of injury resultant from the cellular activation.

Nebulizer Types and Their Function

The nebulizer is a device that takes liquid solutions and converts them into an aerosol. One type of nebulizer is the jet nebulizer. This device consists of the nebulizer console, tubing to pass the airflow, and mouthpiece where the medicine solution is placed. When the machine is activated, compressed air, or another gas, is passed through the tubing from the nebulizer. This gas passes by the solution, draws it into the stream, and breaks it down into aerosol particles. Baffles are used to take any large droplets and return them back to the mouthpiece cup for re-nebulization. This is one of the more common nebulizers used to deliver nebulizer medications to patients. Another type of nebulizer is the vibrating-mesh nebulizer. This nebulizer is similar but uses a different tactic for generating the aerosol. A small mesh plate with small holes is vibrated with electricity. The liquid is passed from a solution reservoir raised above the plate to be broken into aerosol via the vibrating plate. The conventional application in medicine is that the medication is drawn into the attached mouthpiece to be passed to the patient on the inbreath. Even with the different styles of nebulization, both of these serve to generate an easy to inhale form of the medication. This aerosol is delivered directly into the patient's lung tissue and can reach into the secondary small airways. There are multiple different medications that can be delivered via nebulization, but it is important to note those that affect receptor systems involved with a SARS-CoV-2 infection.

Combination Therapy Versus Individual Medications

An example of combination therapy of individual medications can be briefly addressed by looking at Ipratropium Bromide and Albuterol Sulfate. These are medications that come in a mixed form besides their individual treatments. This

medication combo is even more effective at holding a patient's airway capacity and increasing the FEV of COPD patients. This is because the mixed combination acts on both muscarinic receptor and beta-adrenoceptor systems at the same time via antagonism and agonism, respectively. Data shows that patients taking combined therapy had a higher percentage change in FEV than those with just plain albuterol. In addition, a higher percentage of patients in the sample size had an increase of at least 15% in FEV on the combined therapy versus the albuterol. The medications were tested after 15 minutes of the dose and compared to baseline levels at 0 minutes. In addition to the increase in FEV, there were no increases in adverse side effects or worsening of pre-existing conditions. That combination therapy did not result in an increase in adverse effects. In fact, the overall incidence of adverse events was lower in the combination therapy group (25.4%) than in the albuterol group (33.3%). Nor was combination therapy associated with worsening of adverse effects: only 14.1% of patients using the combination aerosol reported moderate to severe adverse events, compared with 22.2% in the albuterol group. These results show that treating COVID patients with these medications would be best done using the combination therapy to promote the maximum opening of the airways with as few side effects as possible. All these nebulizer medications have additional benefits when treating COVID patients since they are dosed using a nebulizer. The essence of this combination therapy thus positively relates to the Bio-Atmosphere within the enclosure chamber of the BioZone Unit having the ability to combine the Bio-Atmosphere treatment regimen.

Benefits of Nebulizer Delivery Versus Inhalers

While these medications are commonly delivered via a nebulizer, they also come in metered dose inhaler (MDI) forms. There are also commonly prescribed as the delivery method for the medications for ill patients with other lung diseases. However, research indicates that the nebulizer has a more effective delivery method than that of MDIs. These devices release an aerosol dose when pressed which is pulled into the patent's lungs on an inbreath. This is very similar to the delivery method to the nebulizer but has some slight drawbacks. A study from Hope Hospital in Salford UK followed patients with airway obstructions over a five-year period to see the long-term effects of using home nebulizers with the previously mentioned medications to study these patients' spirometry as well as a nebulizers comparative benefit to MDIs. The 20 results of O'Driscoll and Bernstein indicated that patients taking the medications through nebulized methods had FEV levels higher above baseline after the study than those that used the MDI.

In addition, the mortality rate amongst the nebulizer patients was slightly lower than that of the MDI patients. Part of this can be attributed to the need for a hand-

to-breath coordination for the treatment. Some patients can struggle with taking an inbreath at the same time the dose is released from the chamber. This can lead to a decrease in the administered concentration of the drug to the patient's airways. Given that a nebulizer produces a continuous stream of aerosol, the patient can breathe normally without the need for coordination. In addition, more medication types can be delivered via nebulizer than with traditional MDIs. However, since aerosol is the primary method for SARS-CoV-2 transmission, it is important to understand how to better administer nebulized medications to infected patients without causing unnecessary viral spread to the surrounding environment. This same discussion applies to the airtight enclosure of spaceships.

A prolonged space travel necessitating one or more astronauts to regularly receive nebulized or MDI treatments for a medical condition. If enough medication escaped into the enclosed atmosphere it is theoretically possible that the medication accumulation could prove harmful to the astronauts or the food and microbiome systems relied upon. In many instances death of asthmatic or COPD patients at home result in the overuse of the inhalers or nebulizers and it has been proven that an excessive amount of the bronchodilators, for example, actually reverse their efficacy and cause bronchoconstriction making it even harder for the person to breathe.

Nebulizers and Aerosol Safety

This section relates to the ability of the BioZone enclosure cabinet to deter and prevent contaminating the environment through the use of breathing treatment devices.

Many are concerned with the potential spread of contagions via aerosol. This brings forth the concern for safety measures in the treatment of COVID patients with nebulizer medications. There is a significant aerosol release into the ambient environment when using a typical Jetstream nebulizer to administer the patients medicine. A journal article in Respiratory Medicine by Dr. Arzu Ari notes that, "Although conventional jet nebulizers are commonly used to deliver aerosolized medications, they may also spew **2/3** of the emitted aerosol into the ambient environment. In this case, healthcare providers are exposed not only the inhaled medications but also to the droplets from the patient's airways and lungs". However, there are ways to combat and protect both healthcare workers and other individuals from the risk of viral transmission. In the same article Dr. Ari goes on to mention that manufacturers of nebulizer kits have developed filters to be placed on the exhalation port of the mouth pieces to cut down on this aerosol release. The article claims that "While the placement of a filter to the nebulizer was 93% effective in capturing exhaled aerosol droplets and will reduce secondhand

exposure of aerosol medication to health care professionals, the efficiency of these filters in preventing the transmission and the magnitude of the risk acquiring coronavirus through filtered nebulizers are not fully known". This is a very stark contradiction to the idea that exhaled aerosols cannot pose a threat to healthcare providers.

Nebulizers and Ventilator Patients

Many patients that develop extreme infections of SARS-CoV-2 tend to develop worse symptoms. The lung system can develop ARDS and leave patients with no capability to breathe. This requires patients to be placed on ventilators. The use of nebulizers attached to patient ventilators can help these patients to decrease fluid and mucus production as well as de-inflame the airways. The location of the nebulizer in the ventilator circuit, as well as the type of nebulizer affects the efficiency of the medication delivery. A study was conducted using a mechanical human lung with both pediatric and adult settings. A circuit with a nebulizer was attached to the ventilator machine at different locations with different types of nebulizers. The circuit was tested with both Jetstream and mesh nebulizer types. The study was conducted by Ari et al. from the division of Respiratory Therapy at the School of Health Professions at Georgia State University. In their study they found that mesh nebulizers at the position placed before a heated nebulizer had the most effective deposition of the dose distal to the endotracheal tube. The study shows that nebulizer medications can be effectively delivered to a ventilator patient with COVID-19 to help alleviate symptoms. The medication used in this experiment was albuterol sulfate and they concluded that using a mesh nebulizer at position two would possibly improve bronchodilatation. They found that it benefits to place the nebulizer at certain positions because it also takes the weight of the machine away from the patient airway which is better for smaller patients. A faster speed of aerosol flow through the circuit showed that less would be taken into the patient's airways, which is why a mesh nebulizer, which has no additional gaseous flow, helps to increase the amount of medication delivered to the patient's lungs. The previously mentioned article by Arzu Ari also stated, "Mesh nebulizers can stay in-line for up to 28 days, and reservoir design allows adding medication without requiring the ventilator circuit to be broken for aerosol drug delivery. Unlike the jet nebulizer, the medication reservoir of mesh nebulizers is isolated from the breathing circuit that eliminates the nebulization of contaminated fluids". This shows a higher level of safety for practitioners taking care of patients in intensive care units as they will have minimal exposure to secondary aerosols from infected patients, although filters that are used with nebulizers are also effective when in circuit. That article mentioned that the amount of drug deposited at the exhaust port was greater than 160-fold higher without the expiratory filter than

with the filter in place. This reference was stated since the BioZone Unit itself has an AI regulated Bio-Atmosphere wherein a preferred enclosure atmosphere is maintained for prolonged utilization without the need for additional personnel constantly attending the respiratory necessities of the person temporarily residing within the Bio-Atmosphere enclosure. Multiple fail-safe provisions are described to continually assure the safety of the person receiving Bio-Atmosphere therapies.

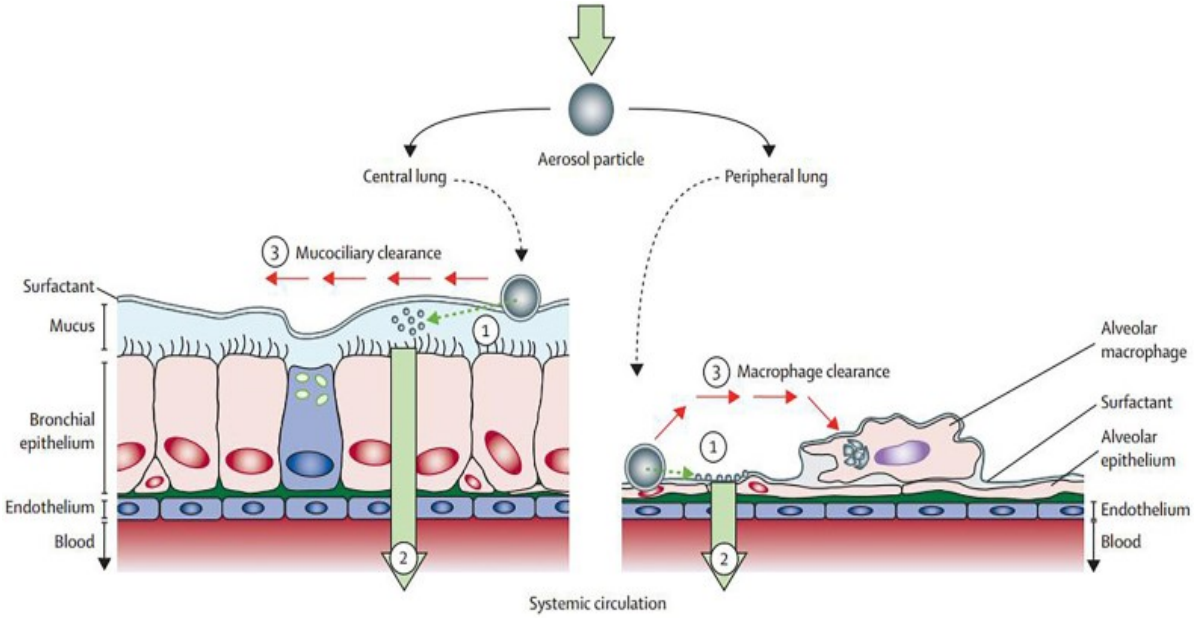


Diagram (8) The fate of aerosol drugs after deposition in the lungs.

(1) Active pharmaceutical ingredient (API) is released from the deposited aerosol particles when these come in contact with the lung lining fluid. Both physicochemical properties of the drug molecular and the formulation forms could control this process. (2) The particle absorption process *via* translocation into the cells, blood and lymph. (3) The particle clearance by the mucociliary escalator and phagocytosis of macrophages.

Alternate Therapy Options for Covid-19 and Their Drawbacks

As stated previously, there is no conclusive treatment option for COVID-19. However, many suggestions have been made as to what mediations can and will improve symptoms. One common treatment option suggested is the use of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). These medications are commonly used in patients that have cardiovascular conditions such as hypertension and heart failure. The idea is that these medications will lower the body's production of AngII and help alleviate inflammation in tissue. The problem with these medications is that they can

actually increase the amount of ACEII receptors in the cardiopulmonary circuit. This gives SARS-CoV-2 more target proteins to bind to and more opportunity to enter host cells. Dr. James Diaz, a Professor of Anesthesiology at Louisiana State University states, “Intravenous infusions of ACEIs and ARBs in experimental animals increase the numbers of ACEII receptors in the cardiopulmonary circulation. Patients taking ACEIs or ARBs chronically for cardiovascular diseases are assumed to have increased numbers of ACE2 receptors throughout their cardiopulmonary circulations as observed in experimental animal models”. In his hypothesis he also goes on to mention that laboratory studies from China have showed that patients with pre-existing conditions treated with ACEIs and ARBs had more chronic symptoms with their SARS-CoV-2 infections.

Another treatment option that has been suggested is the use of beta-adrenergic blockers as a possible treatment for a SARS-CoV-2 infection. These drugs work to halt renin production and inhibit the RAS system cycle. This method of treatment shows promising results as a preventative treatment, as it lowers the amount of ACEII receptors in the pulmonary system and gives less opportunity for spike protein binding. However, it does also shut down and eliminate the ACEII function of breaking down target angiotensin peptides. This means patients that are already experiencing inflammatory symptoms may have a longer timeframe being inflamed as a key component of de-inflammation has been removed. An article in BioEssays written by Natesan Vansanthakumar does show that this treatment type does decrease multiple second messenger inflammatory molecules such as TNF-alpha but fails to touch on the loss of benefit from deactivating ACEII based de-inflammation. While no method of treatment will be completely perfect, we need further investigation and testing in all posed treatment options to gain the best understanding of each one’s benefit.

Patients Already Being Treated with Nebulizer Medications

These target medications have already shown to be effective in treating certain airway diseases. Conditions like COPD and asthma also are currently treated with these medications and these patients can also contract a SARS-CoV-2 infection. Interestingly, patients already being treated with one of these medication types for a pre-existing airway condition appear to be encouraged to continue taking these regimens. Alexzandra Hughes-Visentin & Athea B. M. Paul state that, “However, similar to other coronaviruses, it is also hypothesized that SARS- Cov-2 will precipitate asthma exacerbation. Therefore, further investigation into the immunopathological mechanism still needs to be elucidated to determine risk of severe exacerbations in asthmatic patients. It is for these reasons among others that

it is recommended that asthmatic patients continue their maintenance medications throughout the pandemic". They go on to also mention that combination therapies of long-acting beta-agonist drugs reduce SARS-CoV-2 replication and cytokine production, medications that are already used to treat asthma patients. Asthmatic patients were also shown to have less severe symptoms of COVID contrary to popular beliefs on viral induced exacerbation. "The respiratory epithelial cells in patients with asthma have decreased gene expression for ACE2 receptors and therefore may be protective against COVID-19 infection", Alexzandra Hughes-Visentin & Athea B. M. Paul note. Both the existing airway condition as well as the nebulized medications themselves potential serve to help ease the severity of infection. This shows that while further investigation needs to be done, lesser symptom results of COVID-19 when patients are previously medicated on a nebulizer cannot be ruled out.

XIV. BIOZONE THERAPEUTIC DRUG DELIVERY SYSTEM

Conventional delivery systems for pharmaceutical therapies have included the Parenteral, Transdermal, Mucosal, Oral and Inhalation routes. For our purposes we will be discussing the Inhalation route inclusive of the advantages, disadvantages and theoretical applications thereof. Local administration of therapeutics by inhalation for treatment of lung diseases has the ability to deliver drugs, nucleic acids and peptides specifically to the site of their action and therefore enhance the efficacy of the treatment, limit the penetration of nebulized therapeutic agent(s) into the bloodstream and consequently decrease adverse systemic side effects of the treatment. Nanotechnology allows for a further enhancement of the treatment efficiency.

To better investigate our intended pulmonary delivery system, we must first review modern therapeutic approaches of inhaled nanoscale-based pharmaceutics for the detection and treatment of various lung diseases. Next, we will evaluate promising agents that may be utilized with the BioZone Bio-Atmosphere Therapeutic Drug Delivery System to combat specific disease or injury mechanisms. Finally, we will explore the unique and petitioned proprietary provisions of the BioZone System being developed to significantly enhance and safely administer Pharmaceutical Deliveries through the Pulmonary System.

A. Pulmonary Delivery of Pharmaceuticals; Introduction

Lungs represent an attractive alternative route of drug delivery. They possess a large area for the deposition of therapeutics and high vascularization for the systemic delivery of various pharmaceutical agents. Inhalation lung delivery prevents the degradation of active components in the gastrointestinal tract and first pass metabolism in the liver. Possible high lung toxicity of drugs and their degradation by lung macrophages, the risk of drug-induced lung injury and occupational exposure of health care workers to nebulized drugs have limited enthusiasm for the inhalation route for the systemic drug delivery. On the other hand, the use of systemic delivery of pharmaceuticals for treating lung diseases in most cases demonstrates a low efficiency and potentially severe adverse side effects on other organs.

To enhance the efficacy of the treatment of various lung diseases and limit exposure of healthy organs to potentially toxic drugs, it seems natural to deliver therapeutics directly to the lungs by inhalation. An ideal drug formulation and inhalation delivery method should provide a local inhalation delivery specifically to the diseased cells, limit the exposure of healthy lung cells and restrict drug penetration into the circulation. While several efficient inhalation drug delivery devices have already been developed, tested and implemented in clinical practice, the formulation of efficient drug delivery systems for local targeted inhalation delivery of various therapeutic modalities is still in the developmental stage.

Recent advances in nanotechnology open a door for enhancing the efficacy of inhalation treatment of different lung diseases. The application of nanotechnology to the design of drug delivery systems for effective delivery of therapeutics specifically to tissues and cells affected by the disease allows for enhanced treatment outcomes and prevention of severe adverse side effects upon tissues and cells, including those in the lungs, as well as entire organs. This review briefly describes modern patents related to the inhalation drug delivery and recent clinical trials of therapeutics for treatment of lung diseases.

Inhalation local delivery

The term inhalation local delivery is used in most cases to denote inhalation route of delivering therapeutics or other exogenous entities directly to the lungs with their preferential accumulation in the specific lung areas or cells and limited penetration into the blood circulation. The efficiency of inhalation local delivery mainly depends on lung aerodynamics, breathing conditions, particle size, inhalation methods and devices used. To be inhaled, a delivered liquid or solid

should be suspended in a gaseous medium to form an aerosol our attention on the engineering of nanotechnology-based delivery systems that are being used for the local inhalation delivery of therapeutics to the lungs. Moreover, if the inhaled delivery system is specifically targeted to diseased cells (e.g., cancer cells), then healthy lung cells will also be protected from the drug or other inhaled exogenous substances. An efficient delivery system specifically designed for the local inhalation lung delivery should therefore retain in the lungs or even preferably, specifically in the diseased cells for the required treatment period and not penetrate in its active form into the bloodstream in order to protect the rest of the body from the potentially toxic exposure.

Local inhalation delivery of therapeutics in most cases substantially changes the organ distribution of delivery system and its active components in the organisms when compared with oral or parenteral delivery. In contrast, some types of nanoparticles (liposomes, nanostructured lipid carriers, mesoporous silica nanoparticles, anisotropic polymer/lipid "Janus" particles, etc.) preferentially accumulate in the lungs after inhalation.

Pharmacokinetics

Local inhalation delivery of pharmaceutics directly into the lungs in most cases improves pharmacokinetics of the delivered agent(s). Generally, it increases the retention of the delivered drug in the lungs.

Lung toxicity of drugs

When a drug or other biologically active substance is delivered via inhalation, the lungs are inherently exposed to its action and open a possibility of side effects. This is especially important in case of highly toxic anticancer drugs. In this case, the delivery of toxic chemotherapeutic substances directly into the lungs with limited penetration into the bloodstream may even protect the rest of the body from undesirable toxic effects of the treatment. Drug(s) can be specifically targeted to the diseased cells. Being delivered by inhalation, some drugs can potentially induce lung damage, provoke the development of lung disease or increase the severity of existing ones. It was found that about 400 drugs can potentially induce different lung diseases. In addition to systemic treatment, where personnel can also be exposed during the preparation, injection and disposal of the toxic agent, exhalation of residual amounts of aerosol represents a serious threat to the health of

healthcare workers that routinely perform the treatment procedures. Fortunately, recently developed filters and air cleaning systems substantially minimized such risk. Nevertheless, the possibility of exposure of health workers to the dangerous aerosols should be considered as a hazard during inhalation therapy and minimized by corresponding measures.

Lung defense mechanisms

The lung represents an organ in the human body that is systematically exposed to different and often damaging parenchyma substances. Naturally during evolution, many defensive mechanisms were developed in the lungs in order to limit exposure to potentially dangerous substances and minimize the damage caused to the lung structures. Major lung defensive mechanisms include beating cilia, mucus, macrophages, transporters and enzymes. In addition, manifestations of lung disease(s) and impaired pulmonary function can potentially interfere with inhalation therapy and prevent deposition of aerosols into the desired regions of the lungs or cellular uptake of the therapeutics. Consequently, inhalation devices, regimens and delivery systems should be designed regarding these obstacles. It should be stressed however, that lung defensive mechanisms much less affect the local inhalation treatment of lung diseases when compared with systemic inhalation delivery. Nevertheless, lung defense mechanisms must be taken into account when designing delivery systems for inhalation therapy. In addition to drug(s), such complex systems may include additional components that help to overcome or suppress lung defensive mechanisms. For example, in order to defeat cellular drug efflux transporters that pumps out drugs from the lung cells, suppressors of corresponding proteins (e.g., nucleic acids or small molecules) can be included into a complex delivery system. Similarly, suppressors of cellular anti-apoptotic resistance can also be included in the system in order to enhance cell death induction by anticancer drug(s). It should also be taken into account, that the complexity of the delivery system substantially increases its cost and makes the production substantially more complicated.

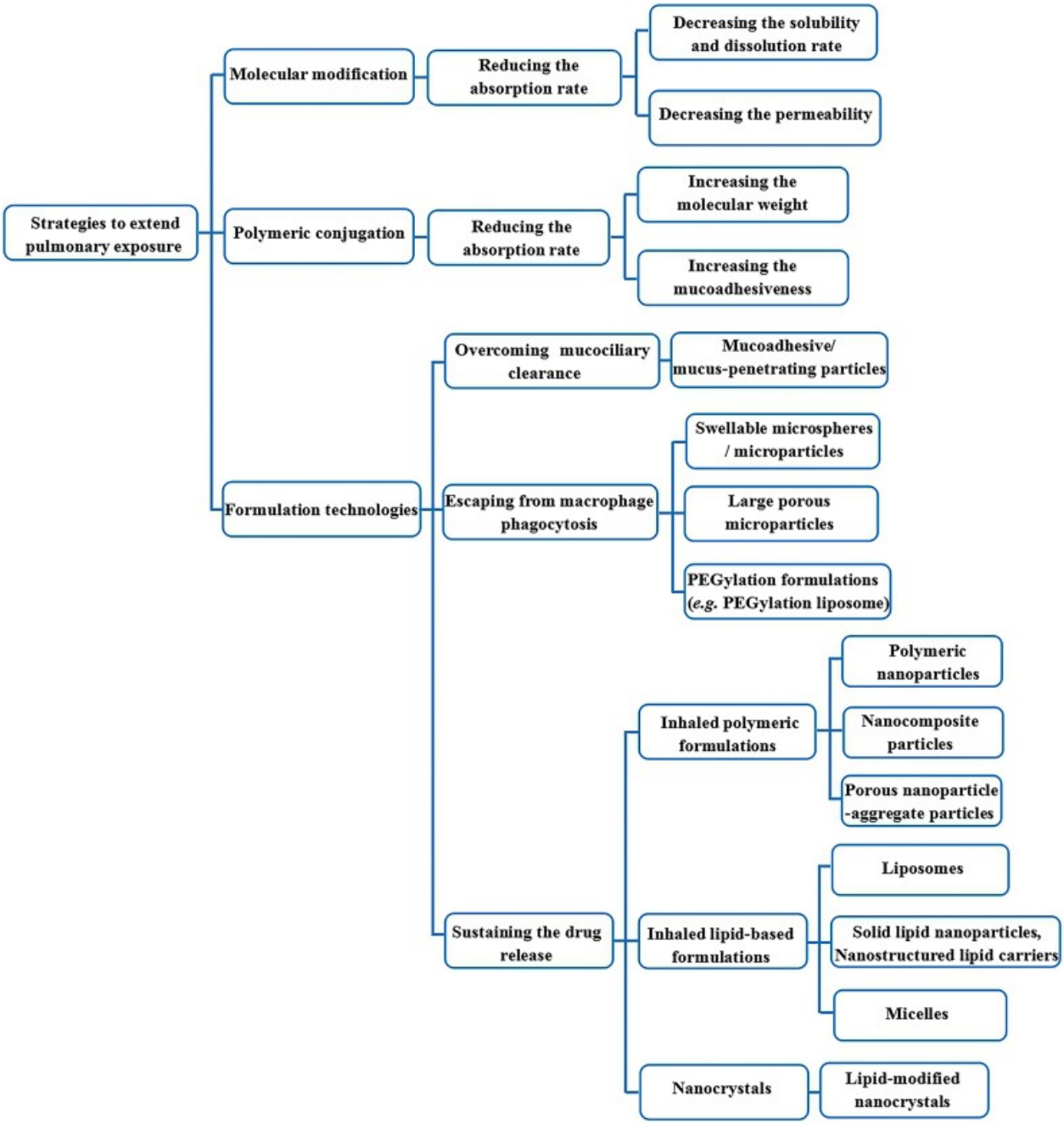
Drug stability

A therapeutic agent should not drop its activity during the process of nebulization and its travel to the site of action. Drug destruction during the nebulization depends on the type of nebulizer and regimen of inhalation. Special constructions of

nebulizers were developed in order to protect the integrity of drug delivery system and prevent drug degradation during aerosol formation In addition, the selection of a right size of delivered aerosol particles helps to target a specific region of the lungs. It is generally believed that aerosol particles with mean diameter of 5–10 μm are preferentially deposited into oropharynx and large conducting airways. In contrast, smaller particles with diameter less than 1–5 μm are deposited in the small airways and alveoli. In particular targeting cell surface receptors, intracellular organelles and molecules may be useful for active targeting in case of inhalation delivery of pharmaceutics. Many different active targeting approaches were developed during the last two decades and tested in experiments and in clinics. One of such active targeting approaches was developed and evaluated in or laboratory and is based on targeting luteinizing hormone releasing hormone (LHRH) receptors. It has been found that LHRH receptors overexpressed in the plasma membrane of various cancer cells (including lung cancer cells) while their expression in healthy cells from visceral organs normally does not exceed the detection level of modern polymerase chain reaction method. Consequently, when the LHRH peptide in its native or modified form is added to the delivery system, the entire system and delivered drug is accumulated predominantly in cancer cells leaving healthy ones intact. Such targeting approach was successfully applied for the inhalation delivery of nanoparticle-based drugs and nucleic acids specifically to lung cancer cells. It was found that non-targeted nanostructured lipid carriers (NLC) were relatively uniformly distributed within the lungs including healthy lung tissues. In contrast, LHRH-targeted NLC predominately accumulated in lung tumor nodules with minimal accumulation of the particles and drugs in healthy tissues. The majority of these types of delivery systems include colloidal dispersions, different microparticles and nanoparticles.

B. Inhalation delivery systems

The following Flowchart depicts current strategies to extend the pulmonary exposure of the inhaled drugs.



Colloidal dispersions

Colloidal system or colloidal dispersion for drug delivery represents a heterogeneous system which consists of a dispersed phase (solid or liquid drug) homogenously distributed within the dispersion medium (usually water). The simplest way to produce aerosols of water insoluble drugs is a dispersion of the solid or liquid hydrophobic drug in water using probe sonication. The resulting colloidal dispersion can be used to produce aerosols by different methods and then delivered into the lungs by inhalation.

Microparticles

The term “microparticle” in drug delivery applications generally refers to a particle with one or several micrometers in size. According to the International Union of Pure and Applied Chemistry (IUPAC), a nanoparticle represents a particle with dimensions between 1 × 10−7 and 1 × 10−4 m. However, it is stressed that the lower limit of the distinguishing between micro- and nanoparticles is still debatable. For our intent, in terms of usefulness for the delivery of drugs, the particles with dimensions lower than 0.5 μm should be referred to as nanoparticles.

Microparticles of many materials including ceramics, glass, polymers, and metals are currently commercially available. However, polymer and metal microparticles are being mainly used for the drug delivery purposes. Paclitaxel loaded alginate microparticles represent a typical example of microparticles designed for inhalation delivery and built using a natural polymer–alginic acid. Alginate represents a biocompatible, biodegradable and mucoadhesive polymer that effectively binds to A.

Nanoparticles

In general, nanoparticle represents particles with sizes at least in one direction smaller than 500 μm. They normally form a stable colloidal dispersion. Currently, nanoparticles are widely used for drug transport via various delivery routes, including inhalation. During inhalation delivery, nanoparticles can form droplets or aggregates with higher micrometer size. However, a major behavior of these particles after the deposition inside the lungs depends on their nanoscale range dimensions. The composition, size and shape of nanoparticles significantly influences their retention in the lungs and targeting properties.

Polymers

Of different composition represent a major part of nanotherapeutics that are used for drug delivery via various routes, Traditionally, we consider as a “polymeric” drug delivery system compositions that have a linear polymer conjugated with active components of the system (drugs, targeting moieties, etc.) directly or via spacers of different architecture. The examples of polymeric systems for inhalation delivery of drugs include poly(lactic-co-glycolic) and poly(ethylene glycol)-co-poly(sebacic acid) aerosol dry powder formulations. Because of the relatively small size, carriers comprised from a “pure” linear polymer can easily penetrate into the systemic circulation and open a door for adverse side effects. Consequently, polymers are mainly used in a complex with other molecules (e.g., nucleic acids or lipids) to form nanoparticle-like structures.

Dendrimers

The term dendrimer (from a Greek word Dendron; tree) usually denote a highly branched structure. The size of most dendrimers used as drug carriers varies from 4 to 20 nm. While this size provides for an efficient internalization by different targeted cells, dendrimers usually rapidly penetrate into the circulation minimizing their retention in the lungs.

Lipid-based nanoparticles

Lipid-based nanoparticles are used extensively for various drug delivery applications. These nanocarriers allow for easy incorporation of lipophilic drugs in its lipid core/membrane. The amphiphilic nature of many lipids allows them to form various structures and incorporate hydrophilic drug molecules as well. In addition, lipid carriers can be made from biocompatible lipids similar to those that comprise cell membrane. This not only limits toxicity of lipid carriers but allows them to easily penetrate inside different cells.

In terms of inhalation lung delivery, lipid particles can be easily aerosolized and usually are well taken by the lungs providing for a prolonged retention of carriers and drugs in the lungs.

Lipid nanoparticles

It seems natural to choose lipids similar to those contained in lung surfactants in order to form carriers for inhalation lung delivery. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine-methoxy(polyethyleneglycol) (DPPE-PEG) are often used for these purposes.

Liposomes

Liposomes probably are the most widely used and best characterized lipid-based drug carriers. In most cases, a typical liposome consists of a single bilayer lipid membrane (unilamellar liposomes) or several bilayer lipid membranes (multilamellar liposomes). The outer surface of liposomes is often modified by polymers (mainly poly(ethylene glycol), PEG). Such coating performs several functions. It adds STEALTH properties to the liposomes and allows for conjugating additional components of the delivery system (e.g., targeting moiety) to the distal end of the polymer. In most cases liposomes suitable for drug delivery have a size range of 50–500 nm, while larger size liposomes have also been employed. It also should be stressed, that by varying a composition of liposome membrane(s), a neutral, negatively charged and cationic liposomes may be created. The later liposomes can be used to form complexes with negatively charged

nucleic acids. In most cases, after carefully selecting liposome composition and methods of liposome nebulization, liposomes preserve their size, payload and do not aggregate after aerosolization. They demonstrate a preferential accumulation in the lungs, suitable lung retention for an extended period, penetration into lung cells after inhalation, and release of active payload inside the cells. In most cases, no significant adverse side effects were registered after the application of neutral or slightly negatively charged liposomes. However, cationic liposomes were found to be toxic to human cells and potentially can introduce genetic aberrations. Moreover, adverse side effects of cationic liposomes significantly increased with an increase of positive charge of the particles. However, because cationic liposomes normally are used for the formation of almost neutral complexes with negatively charged nucleic acids, such modification of cationic carriers usually prevents adverse effects on the cells.

In addition to modification of different types of lipid nanoparticles, polymers can be used to form lipid–polymer hybrid nanoparticles as an alternative to other lipid based nanocarriers including liposomes. Poly(lactic-co-glycolic acid) nanoparticles enveloped by phosphatidylcholine (PC) or PC stearylamine layers were tested as lipid–polymer hybrid nanoparticles for inhalation delivery. The resulting nanoparticles were spherical in the shape and were adsorbed onto the carrier chitosan particles. However, the suggestion that nanoparticles can be exhaled and not deposited in the lung tissues and cells contradict an extensive literature data showing that lipid-based nanoparticles with the size smaller than 1 μm are successfully deposited and retained in the lung tissues and also penetrate into the lung cells. Another hybrid polymer/lipid nanoparticles, suitable for inhalation lung delivery so called **“Janus”** nanoparticles. These nanoparticles have two distinct phases.

Nanostructured lipid carriers (NLC)

Represent a new generation of lipid nanoparticles suitable for inhalation delivery of different drugs and siRNA. NLC are prepared from mixtures of solid (e.g., Precirol ATO 5) and spatially incompatible liquid lipids (e.g., Squalene) by melt-emulsification. Lipophilic drug can be loaded into the inner core of NLC. Such cationic nanoparticles can form complexes with negatively charged nucleic acid molecules. Alternatively, thiol-modified DNA or RNA molecules (e.g., small interfering RNS, siRNA) can be conjugated to the surface of NLC via biodegradable disulfide (S–S) bonds.

Nanospheres

A novel class of synthetic carriers designed mainly for gene delivery was proposed. These nanospheres were synthesized using four poly-ethyleneoxide/polypropylenoxide blocks centered on an ethylenediamine moiety. The nanoparticles had a positive charge and easily formed complexes with nucleic acids/plasmids. Later, these nanospheres were successfully used for inhalation delivery of the chemokine fractalkine as a cancer chemotherapeutic agent. Two generic approaches are usually used for delivery of nucleic acids using non-viral vectors. The first approach includes a complex formation between negatively charged nucleic acid molecules and positively charged carrier materials. As the result of the complex formation, positively charged nanoparticles are formed. The second approach for the delivery of nucleic acids comprises a direct conjugation of a modified nucleic acid molecule to the carrier via a biodegradable chemical bond. Polyethylenimine (PEI) and its derivatives as well as DOTAP are most widely used carriers for gene delivery. In addition, practically all types of cationic carriers are suitable for complexation with nuclei acids and their inhalation delivery. In particular, glucosylated polyethylenimine, chitosan, spermine-altpoly (ethylene glycol) polyspermin, polypropylene Imine (PPI), silica and other substances have been successfully used to form nanoparticle carriers for inhalation delivery of nucleic acids.

Magnetic nanoparticles

Magnetic nanoparticles can be used for imaging and drug delivery. When such particles accumulate in the targeted site they can be used as contrast agents for magnetic resonance and other contrast imaging. These nanoparticles can be targeted to the site of action by an external magnetic field. In addition, the application of high intensity external magnetic fields raises the temperature of such nanoparticles comprised mainly of metals. High temperature can be used for killing targeted cells (e.g., foci of bacterial infection or tumor nodules). The application of magnetic nanoparticles for inhalation local lung delivery has also been proposed and evaluated. A simple superparamagnetic iron oxide nanoparticles (SPIONs), or more complex surface coated of Fe_3O_4 magnetic nanoparticles with polymer poly(lactic-co-glycolic acid) and lipid-coated SPIONs have been used for these purposes. Below we discuss major therapeutic applications of inhalation delivery.

Asthma and chronic obstructive pulmonary disease (COPD)

Treatment and management of asthma and COPD is the most known application of inhalation delivery of therapeutic agents. Various types of β agonists,

anticholinergics, corticosteroids anti-inflammatory drugs are effectively delivered by inhalation.

Lung hypoxia and edema

Tissue hypoxia accompanies many lung diseases (lung edema, pneumonia, fibrosis, etc.), aggravates the primary disorder, and causes additional cell damage. It was found that the major mechanisms of anti-hypoxic action of liposomal α tocopherol counter tissue hypoxia. It should be stressed that liposomes used in this study were comprised with phosphatidylcholine, a major component of lung surfactant system. Consequently, delivery of this phospholipid normalized at least in part its deficiency caused by severe hypoxia. This in turn improved lung biomechanics, breathing pattern and increased oxygen consumption. Inhibition of lipid peroxidation by the supplied vitamin E decreased hypoxic damage of air–blood barrier and also limited cellular damage caused by oxygen reactive species. Taken together all these factors limited hypoxic cellular damage, lactate-acidosis, and prevented cell death from apoptosis and necrosis. Consequently, the resistance of an entire organism against acute severe hypoxia significantly increased. As a result, the mortality of animals with hypoxia was substantially decreased after the treatment with liposomal α tocopherol.

Lung injury

Potentially, inhalation delivery of drugs can help to minimize lung injury caused by the damaging environmental impacts. For instance, it was shown that inhalation delivery of kinase-deficient Akt1 gene that encodes one of serine/threonine-protein kinases attenuates injury of secretory Clara cells induced by naphthalene. These types of cells function in innate defense and epithelial repair.. In most cases, immunosuppressive agents are delivered systemically, mostly by parenteral injections. However, in case of lung transplantation, it is logical to deliver such agents directly to the lungs in order to suppress lung transplant rejection and limit adverse side effects on the overall body immunity.

Fungal infections

The risk of lung fungal infections increased in patients undergoing chemotherapy, organ and cell transplantation or treated in intensive care units. However, pulmonary infections often poorly respond to the systemic treatments due to the low accumulation of antifungal drugs in the lungs. Several attempts were made in order to deliver antifungal drugs via inhalation. A 2-hydroxypropyl-β-cyclodextrin (HPβCD) solubilized itraconazole (ITZ) solution (i.e., HPβCD-ITZ) and colloidal dispersion of ITZ were created and delivered as aerosol to mouse lungs. The

analysis of pharmacokinetics and distribution pattern showed that both formulations are suitable for local inhalation delivery to the lungs. In another independent study, aerosolized commercially available voriconazole solution was tested for the prevention of invasive pulmonary aspergillosis. The results showed clear advantages of voriconazole delivered via inhalation over amphotericin B deoxycholate delivered intraperitoneally.

Pulmonary fibrosis and inflammation

Idiopathic pulmonary fibrosis (IPF) often accompanied by inflammation is a devastative lung disease that is often associated with mortality. Treatment of this disease is difficult because an effective therapy is not available yet. It has been proposed to use liposomal form of prostaglandin E2 for treatment of IPF via inhalation. The formulation was tested on experimental mouse model of IPF and inflammation caused by intratracheal administration of bleomycin. It was found that treatment of animals with such a liposomal formulation decreased the signs of IPF, blocked overexpression of many proteins involved in the development of IPF, inflammation and fibrotic lung injury and finally prevented animal mortality.

Most existing systemic therapies (administered by intravenous or oral routes) are not very effective for the treatment of primary lung cancer and metastases and/or induce severe adverse side effects. Inhalation (local drug delivery) would be an important part of combination therapy together with systemic or local treatment of lung cancer, especially of its metastases to other organs. Consequently, local pulmonary inhalation delivery of anticancer agents potentially can improve the outcome of lung cancer therapy.

Correction of hypoxic lung damage by liposomal α tocopherol.

Conventional anticancer drugs in forms that are ready for aerosolization to various types of antibodies and nucleic acids, drug that activates cellular immune response, delivery systems for hyperthermia and radiotherapy, adjuvant inhalation chemotherapy and combinational therapy.

Antibodies

Antibodies represent an attractive alternative to traditional chemotherapy with anticancer drugs. By their nature, they are targeted to the specific site of the action and potentially can provide a more effective treatment and fewer side effects. Several monoclonal antibodies including cetuximab and bevacizumab targeted to the epidermal growth factor receptor and vascular endothelial growth factor receptor, respectively, have been approved for treatment of lung cancer. Consequently, attempts have been made to deliver antibodies via inhalation in

order to treat lung cancer. An innovative Respite™ system that employs surface acoustic waves (SAW) was developed and tested for inhalation delivery of monoclonal antibodies against the epidermal growth factor receptor. It was found that a portable SAW nebulizer was able to generate an aerosol and did not cause antibody fragmentation or their specific activity.

Nucleic acids

Nucleic acids are currently widely used for the treatment of various diseases, including lung cancer. Viral and non-viral vectors are being used to suppress oncogenes and/or genes responsible for the progression of tumor growth or to overcome cancer cells resistance to chemotherapy. The suppression of oncogenes usually is not very efficient in terms of the suppression of tumor growth. Alternatively, the suppression of genes responsible for the tumor growth and proliferation already generated positive results. Inhalation gene therapy was studied mainly for the delivery of tumor suppressor genes, anti-vascular endothelial growth factor (VEGF), epidermal growth factor suppressor (EGF), K-Ras, and immuno-therapy. Polyethyleneimine (PEI) and its derivatives as linear or branched polymers are frequently used for these purposes. The advantages of PEI include efficient attachments to the airway epithelial cells and introduction of nucleic acids in the cells and their nuclei, protection of nucleic acid molecules from degradation and sheer forces during nebulization. Sometimes PEI-DNA complexes are modified with PEG. Liposomes and other polymers also are frequently used for DNA complexation to form stable nanoparticles. Activation of natural human immune defense system in order to kill cancer cells represents a promising alternative to chemotherapy. It was found that aerosol interleukin-2 induces natural killer cell proliferation in the lung and improved the survival of mice with osteosarcoma lung metastasis. This method of induction of natural killer cells demonstrates advantages over the transfusion of natural killer lymphocytes where infused cells only temporary reside in the lungs.

Hyperthermia

An induction of hyperthermia specifically in tumor cells denotes another alternative to chemotherapy for the treatment of cancer. A main idea of such therapy includes the delivery to the tumor vicinity substances (in most cases metal nanoparticles). However, the fear of decomposition of the treated tumors under the action of hyperthermia, invasion of cancer cells into the bloodstream and development of metastases limit the enthusiasm to this approach. For instance, a combination of intravenously injected human natural killer cells and interleukin-2 delivered as aerosol displayed a synergic effect and substantially enhanced a

survival of mice with osteosarcoma lung metastases. A combination of gene therapy expressing ABC10 protein with aerosol delivery of cisplatin was also investigated. Nanotechnology approaches have been successfully used to improve tumor imaging and radiosensitization. When imaging is combined with cancer therapy, the agent that allows for this combination is usually called a theranostic (or theragnostic) agent that combines the abilities to detect/image cancers with therapeutic effects. Gadolinium based nanoparticles were developed as a theranostic agent for the detection and radiosensitization of lung tumors after their inhalation delivery. It was shown that the use of these nanoparticles localized in tumor nodules of experimental animals and dramatically increased the survival of animals after radiation treatment.

Another approach for a targeted delivery of a contrast agent directly to lung tumor cells was developed in the laboratory. The method is based on the use of PEGylated water soluble Mn3O4 nanoparticles and nanostructured lipid carriers targeted to the lung tumor cells by the LHRH peptide. Inhaled granulocyte macrophage colony-stimulating factor (GM-CSF) GM-CSF is a growth factor capable of stimulating the differentiation of hematopoietic cells to increase the production of neutrophils, macrophages, and dendritic cells. It can also activate established granulocytes and macrophages. Through its immune activating effects, it is believed GM-CSF could be used to effectively combat tumor growth. A number of early phase clinical trials have reported the use of inhaled GM-CSF in the treatment of lung metastases.

Inhaled recombinant human interleukin-2 (rhIL-2) rhIL-2

Is a chemokine approved for the treatment of metastatic renal cell carcinoma and metastatic melanoma. It is believed that this interleukin can enhance lymphocyte cytotoxicity, increase the effects of lymphocyte activated and natural killer cells, and increase interferon gamma production. In the past ten years, there have been a number of clinical trials that have evaluated the effectiveness of nebulized doxorubicin, gemcitabine, and carboplatin. G. Otterson et al. reported the results of a dose escalation study using doxorubicin nebulized via an OncoMyst device which consisted of a Pari LC Plus nebulizer (PARI Respiratory Equipment, Inc.) contained within a system to capture stray aerosols. The droplet size produced by this system was reported to be 2–3 μm in size allowing for effective deep lung delivery.

Inhaled drug-loaded nanoparticles

There have been relatively few clinical trials evaluating the use of inhaled nanoparticle systems for the treatment of lung malignancies. The few trials that have been reported have focused on the use of either liposomal cisplatin or 9-nitro-20-camphothecin Both viral and non-viral delivery systems were investigated. Two clinical trials of adeno-associated virus (AAV) carrying cystic fibrosis transmembrane conductance regulator (CFTR) cDNA have shown safety but limited efficacy data. Early trials using cationic liposomes and DNA plasmids delivered to nasal epithelium showed mixed results.

XV. Specific Agents and Mechanisms

Glucose Metabolism

As previously discussed, many of the people dying in the novel coronavirus pandemic appear to be harmed more by their own immune system than by the virus itself. The infection can trigger a cytokine storm; a surge in cell-signaling proteins that prompt inflammation, that hits the lungs, attacking tissues and potentially resulting in organ failure and death.

Scientists have long known that viral infections can affect human cellular metabolism, the system of biochemical reactions needed to provide energy for everything cells do. Infection with an Influenza A virus sets off a chain of cellular events, or a pathway, that boosts the metabolism of glucose. This action, in turn, triggers the production of an avalanche of cytokines. Blocking a key enzyme involved in the glucose pathway could be one way to prevent a deadly cytokine storm. This connection could explain why people with diabetes are at a higher risk of dying from the virus.

When a virus infects a cell, it steals resources in order to make copies of itself. Infected cells have to boost their metabolism to replenish these resources, and healthy cells must also do so in order to mount an effective immune response. Research has shown that an Influenza A infection increases the metabolism of glucose, the sugar molecule that fuels most cellular activities. The pathway, involving a signaling protein called interferon regulatory factor 5 (IRF5), in which a flu infection can lead to a cytokine storm. During such an infection, high levels of glucose in the blood cause an enzyme called O-linked β-N-acetylglucosamine

transferase (OGT) to bind to, and chemically modify, IRF5 in a process known as glycosylation. This step enables another chemical modification, called ubiquitination, that leads to a cytokine inflammatory response.

The findings suggest that interfering with this pathway could be one way to prevent the cytokine storm seen in flu and other viral infections. Such an intervention would need to be done carefully, however, to avoid shutting off the body's ability to fight the virus altogether. It could be relevant to interfere with glucose metabolism using chemical inhibitors and modulate the cytokine production, but it needs to be said that energy metabolism is essential for our immune cells to fight a virus. It may be important to combine antiviral treatment and metabolic inhibitors, suppressing the virus and reducing the overshooting immune reaction at the same time.

The OGT enzyme involved in this pathway is required to initiate the host's stress response to a viral infection. The initial point of this stress response is to build up an antipathogen immune response and try to fight against virus, but if the inflammatory response keeps going, it will cause collateral damage.

Given the role of glucose in the pathway, could a person's diet have an effect on his or her response to a viral infection? That's a very good question. What scientists do know is that people with type 2 diabetes are more susceptible to severe flu infections. But that risk is not because they have higher glucose levels in their blood. The real reason, Wen says, is that they cannot use glucose effectively, and thus cannot initiate a proper antiviral response. Ultimately, the hope is that by interfering with this glucose metabolism pathway, we might be able to stave off the deadly cytokine storms seen in severe cases of flu, COVID-19, RILI, and or the gamut of additional medical disorders that potentially lead to devastating cytokine storms.

The BioZone Project is now investigating cellular metabolism as a countermeasure intervention to thwart the Cytokine Storm immune cascade and whether a specific diet may prove beneficial in protecting against the effects thereof.

Cytokine Changes

TGF-β

As a powerful pro-fibrotic growth factor, TGF-β has some functions, including inducing proliferation and differentiation of fibroblasts, promoting synthesis of collagen by fibroblasts, inhibiting synthesis of collagenase and plasminogen

activator, and aggregating considerable amounts of inflammatory cells and cytokines (PDGF, TNF-α, IL-4, etc.). Increased TGF-β levels after radiotherapy accompanies elevated collagen IV gene expression, which leads to septal thickening and is indicative of microvascular injury. By stimulating metalloproteinase inhibitors (TIMPs), TGF-β inhibits collagen catabolism, which results in collagen accumulation and conversion of fibroblasts into myofibroblasts, leading to lung architecture remodeling. Increased fibroblasts with the loss of functional cells lead to loss of respiratory capacity, tissue atrophy, and necrosis. Such application may prove advantageous for mitigating the effects of radiation injuries sustained from cancer therapy or space travel.

IL-4 and IL-13

IL-4 is mainly expressed by Th2 cells, and also secreted by innate immune cells including mast cells, basophils, and eosinophils. IL-4 and IL-13, as important pro-fibrotic cytokines, are required for the initiation and maintenance of pulmonary fibrosis, as well as other organs (liver, skin).

IFN-γ

IFN-γ is produced by Th1 cells and plays a key role in anti-fibrosis and immunomodulatory effects. IFN-γ can induce M1 macrophages to express a high level of inflammatory cytokines, such as IL-2, IL-23, and nitric oxide (NO), and promote inflammatory processes. In addition, related research has reported that IFN-γ has anti-fibrotic effects.

PGE2

Is a pro-inflammatory mediator in a variety of diseases. It can inhibit the secretion of TGF-β and the differentiation of fibroblasts into myofibroblast in lung tissue to effect anti-fibrotic activity.

PGE2 are classified as an endogenous negative regulator which drives the activation of lung fibroblasts to inhibit radiation-induced fibrosis, including decreasing fibroblast chemotaxis, fibroblast proliferation, fibroblast growth factor receptor expression, collagen synthesis and myofibroblast differentiation, and increasing collagen degradation.

Glucocorticoids

Suitable doses and treatment with Glucocorticoid (GC) drugs are often used to heal RILI patients, which has been confirmed by multiple studies. GCs exert inhibitory effects on a series of pro-inflammatory genes including cytokines, chemokines, and receptors. As powerful anti-inflammatory and immunosuppressive drugs, long-term and high-doses use of GCs will lead to a high incidence of adverse effects. Also, dosage, dosing regime, specific drug used, and individual patient variability are also important influencing factors. Inappropriate application of GCs could

cause various systemic disorders, including cardiovascular system, central nervous system (CNS), eye, immune system, skin, and others. Common side effects of GCs are glaucoma, cataracts, tissue atrophy and extended wound healing, adrenal suppression, and osteoporosis. During the application of GCs antibiotics should be used if infection is suspected. Other adjutant therapies including oxygen inhalation, atomization, and nutritional support should be applied.

Other than GC drugs, other new drugs used to treat RILI have also been reported. Some reports have noted that mesenchymal stem cells (MSCs) can prevent lung fibroblasts from myofibroblasts to inhibit lung fibrosis. Human umbilical cord MSCs and Hepatocyte growth factor has been studied. PGE2 can inhibit the synthesis of collagens by elevating intracellular cAMP levels and inhibit activation and proliferation of fibroblasts.

Pentoxifylline

Pentoxifylline is an ethyl xanthine derivative that has been demonstrated to exert a prostacyclin-like effect by inhibiting platelet aggregation and enhancing microvascular blood flow. Some studies have also shown that Ptx can promote immunomodulatory and anti-inflammatory activations by inhibiting TNF and IL-1. Ptx combined with alpha-tocopherol [vitamin E (Vit E)] in the treatment of lung fibrosis after radiation shows promise.

Azithromycin

Azithromycin as a macrolide antibiotic, exhibits immunomodulatory and anti-inflammatory effects. Azithromycin inhibits inflammatory signaling by suppressing the expression of lipopolysaccharide (LPS)-induced macrophage-driven chemokines. Azithromycin also has a different influence on cell levels including promoting macrophage polarization, inhibiting the effects of neutrophils, and inhibiting autophagosome clearance.

Angiotensin-converting enzyme inhibitors

(ACEIs) can release pulmonary collagen deposition and fibrosis in the process of radiation. Amifostine, as a radioprotective agent, reduces irradiation-induced DNA damage by eliminating superoxide dismutase 2 (SOD2) and lowers the oxygen concentration around normal tissue.

Neupogen and Neulasta

Both are radiomitigators under the recombinant growth factor grouping and work by granulocyte colony stimulating factor (G-CSF) and have now been approved under animal rule for acute radiation syndrome by the FDA.

A study has observed that radiotherapy activates innate and adaptive immune responses by exposure to immunogenic molecules, thus releasing damaged-

associated molecular patterns (DAMPs) including uric acid, S100 protein, adenosine triphosphate (ATP), high mobility group protein B1 (HMGB1), or tumor antigens. A similar result is expected of radiation exposure in space. RILI is therefore the result of inflammatory cells and chemokines, which has been affirmed in numerous studies. A similar assertion can be made for the pathogenicity of certain virions such as Covid-19.

XVI. BioZone Bio-Atmosphere Provisions

A comprehensive analysis has been presented herein describing the Pulmonary System's anatomy and microanatomy, microcirculation, oxygen dissociation process, and respiratory membrane interface. Next we investigated and delineated the Immune System Inflammatory Responses and Cytokine Storm Event and the association with severe Covid-19 Infections. We then addressed Radiation Induced Lung Injuries (RILI) and compared similarities in the destructive cascades involved between Covid-19 and RILI manifestations. Finally, we discussed therapeutic aerosolization and the pros and cons of Pulmonary Pharmaceutical Delivery Systems and the researched correlative agents utilized.

The BioZone Bio-Atmosphere provides a novel extended exposure pulmonary delivery enclosure and system utilizing, but not limited in scope to; magnetic, harmonic resonance, ultrasonic, ionic, temperature gradient, pressure gradient, viral and non-viral pharmaceutical coupling inclusive of the SARS-2 COVID-19 Coronavirus, nanotechnology immunomodulation, dithermal stimulation, and musical tone modulation, for example. The provisions are therein utilized in a safe and proprietary enclosure system with redundant failsafe mechanisms to prevent contamination of the external treatment area. The applications of the BioZone Bio-Atmosphere are vast and promising and deserve further investigation and maturation.

For a complete analysis of the BioZone System and applications please refer to the BioZone Presentation Document provided on the informational website.