Université de Montréal

Comparative safety respiratory pharmacology: Validation of a head-out plethysmograph – pneumotachometer testing device in male Sprague–Dawley rats, Beagle dogs and Cynomolgus monkeys

par

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Mémoire présenté à la Faculté de médecine vétérinaire
en vue de l'obtention du grade de
maître ès sciences (M. Sc.)
en sciences vétérinaires
option biomédecine

Avril 2010

Université de Montréal
Faculté de médecine vétérinaire

Ce mémoire intitulé

Comparative safety respiratory pharmacology:
Validation of a head-out plethysmograph – pneumotachometer testing device in male Sprague–Dawley rats, Beagle dogs and Cynomolgus monkeys

présenté par

Margarita S. Legaspi

a été évalué par un jury composé des personnes suivantes :

Sophie Cuvelliez, présidente-rapporteuse

Éric Troncy, directeur de recherche

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RÉSUMÉ

Le but de cette étude était d'évaluer les qualifications de performance du système FlexiWare® chez le rat male Sprague Dawley et le singe Cynomolgus éveillés, ainsi que chez le chien Beagle éveillé et anesthésié, suite à l'administration de produits ayant une activité pharmacologique connue. Les produits utilisés incluaient l'albutérol administré par inhalation, la méthacholine, et le rémifentanil administrés par voie intraveineuse. Une solution saline administré par voie intraveneuse, a été utilisée comme substance témoin. Différentes variables ont servi à évaluer la réponse des animaux (rats, chien, singe). Ces dernières comprenaient la fréquence respiratoire (RR), le volume courant (TV), la ventilation minute (MV). Des paramètres additionnels ont été évalués chez le rat, soit les temps d'inspiration (IT) et d'expiration (ET), le temps du pic de débit expiratoire, les pics de débits inspiratoire et expiratoire, le ratio inspiratoire:expiratoire (I:E), le ratio inspiratoire sur respiration totale (I:TB), et l'écoulement expiratoire moyen (EF_{50}).

Les résultats obtenus ont démontré que le système FlexiWare® était suffisamment sensible et spécifique pour dépister, chez les espèces animales utilisées, les effets bronchodilateur, bronchoconstricteur et dépresseur central des substances testées. Il pourrait faire partie des méthodes (ICH 2000) utilisées en pharmacologie de sécurité lors de l'évaluation de substances pharmacologiques sur le système respiratoire des animaux de laboratoire. Les espèces animales utilisées ont semblé s'adapter aisément aux procédures de contention. Les paramètres évalués, RR, TV et MV ont permis de caractériser la réponse des animaux suite à l'administration de produits pharmacologiques à effets connus, judicieusement complétés par les variables de débit. L'ajout de paramètres du temps n'était pas primordiale pour détecter les effets des drogues, mais offre des outils complémentaires d'interpréter les changements physiologiques. Cependant, chez le rat conscient, la période d'évaluation ne devrait pas s'étendre au-delà d'une période de deux heures post traitement.

Ces études constituent une évaluation des qualifications de performance de cet appareil et ont démontré de manière originale, la validation concurrentielle, en terme de précision (sensibilité et spécificité) et fiabilité pour différentes variables et sur différentes espèces.

MOTS CLÉS :

Respiration, Sécurité, Pharmacologie, Rats, Chiens, Singes, Validation.

ABSTRACT

The aim of this study was to evaluate the performance qualifications of the FlexiWare® system in conscious Sprague-Dawley rats, Cynomolgus monkeys, as well as awake and anesthetized Beagle dogs following the administration of pharmacological substances with known effects on the respiratory system. The pharmacological substances included albuterol administered by inhalation; methacholine and remifentanil, both administered intravenously. A preparation of saline solution administered intravenously was used as control. Respiratory monitoring included: respiratory rate (RR), tidal volume (TV), minute ventilation (MV), in rats, dogs and monkeys. Additional time-, flow-, and ratio-derived variables were used in the rat model. Those variables included inspiratory (IT) and expiratory (ET) times, time to peak expiratory flow, peak inspiratory and expiratory flows, mid-tidal expiratory flow (EF50), inspiratory:expiratory (I:E) and inspiratory to total breath (I:TB) ratios.

The results of this study have proven that the FlexiWare® was a reliable method and should be considered in the core battery recommended in safety pharmacology studies (ICH 2000) to assess the broncho-dilative, -constrictive, and central depressant effects of drugs on the respiratory system of the common laboratory animal species. The animals appeared to adapt well to the restraint unit. The variables evaluated, particularly RR, TV and MV, were adequate and allowed to characterize the response of the animals following the administration of the pharmacological substances. They are judiciously completed with flow-derived variables. The addition of within-breath time parameters was not primordial to detect drug effects but offered complementary tools to interpret physiological changes. However the evaluation period should be limited to the first 2 hours post treatment.

These studies represent a performance qualifications evaluation of the system and have originally demonstrated the precision (sensitivity and

specificity) as well as repeatability for different variables and on different species of interest.

KEYWORDS:

Respiratory, Safety, Pharmacology, Rats, Monkeys, Dogs, Validation

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LIST OF ABBREVIATIONS

AAALAC:	Association for Assessment and Accreditation of Laboratory Animal Care International.
CO_2:	Carbon dioxide
DL:	Deciliter
Vd:	Dead space
EF_{50} :	Mid-tidal expiratory flow
ERV:	Expiratory Reserve Capacity
ET:	Expiratory Time
FDA:	Food and Drug Administration
FEV:	Force Expiratory Volume
FRC:	Functional residual capacity
FVC:	Forced vital capacity
H^+ :	Hydrogen Ion
H_2CO_3:	Water carbonic acid
HCO_3^- :	Bicarbonate ion
Hg:	Mercury
IACUC:	Institutional Animal Care and Use Committee
IC:	Inspiratory Capacity
ICH:	International Conference of Harmonization
I:E:	Inspiratory:Expiratory ratio
IND:	Innovative New Drug
IRV:	Inspiratory Reserve Volume
IT:	Inspiratory Time
I:TB:	Inspiratory to total breath ratio
KPA:	KiloPascal
Ml:	Mililitre
MV:	Minute volume
N_2:	Nitrogen dioxide

O_2:	Oxygen
$PaCO_2$:	Partial pressure of carbon dioxide in arterial blood
Pae:	Airway Opening Pressure
PaO_2:	Partial pressure of oxygen in arterial blood
PCO_2:	Partial pressure of carbon dioxide
PEK:	Peak Expiratory Flow
Ppl:	Pleural cavity pressure
Pes:	Esophagus Pressure
PO_2 :	Partial pressure of oxygen
RBC:	Red blood cells
RR:	Respiratory rate
RV:	Residual volume
TLC:	Total lung capacity
TV:	Tidal Volume
VC:	Vital Capacity

DEDICATION

To Fernando,

For his support when I felt vulnerable, for his wonderful and remarkable pure soul. For always be hand in hand and met life's changes and challenges. For built this beautiful life, for the true love we share.

To my parents,

Who showed me the true meaning of life, and truly gave me love and guidance.

To my sister Mariana,

Thank you for always being and standing beside me even when we are far.

To my family,

For their support and patience. Thank you for always believe in me and love me the way I am. Thank you to always be there.

To life,

Which lets me experience good and bad moments, and gives me the opportunity every day to try to become a better person.

ACKNOWLEDGEMENTS

The author would like to thank:

Pr. Eric Troncy, for his support and guidance during this process.

Dr. Simon Authier, for his motivation and coaching.

Dr. Jean-Paul Descôteaux, for his confidence in my capacities.

Pr. Sophie Cuvelliez, for having accepted to chair this jury.

LAB Research Inc, for the support in the realization of this study.

LAB Veterinary Services personnel for their assistance and support in the process of the experiments.

INTRODUCTION

In the drug development process, the testing of new drugs in laboratory animals is a prerequisite (Jasso 2002). The objective of this evaluation is to quantify the effects of potentially new drugs on different animal physiological functions in order to assess their safety. It is recommended to test on two different animal species, one rodent species, normally the rat, and one non-rodent species, either the dog or a non-human primate (Wallas 2001).

The International Conference of Harmonization (ICH) (2000) was responsible to define the guidelines used in safety pharmacology studies. Those studies are part of pharmacology studies, which are divided into three categories: primary pharmacodynamic, secondary pharmacodynamic and safety pharmacology studies (FDA 1997). The term "Safety Pharmacology" was first defined at the ICH in 2001 (ICH 2001). The aim of this discipline is to characterize the pharmacokinetic / pharmacodynamic relationship (PK / PD) of drug's effects using continuously evolving methodology (Pugsley *et al.* 2008).
Pharmacology studies have been considered an important component in drug safety assessment. The reason is that numerous examples are reported in the literature indicating that conventional preclinical toxicity testing methods could not predict problems occurring following the administration of new dugs to patients. Some of the problems are hepatotoxicity (Peters 2005) and central nervous system effects (Tsaioun *et al.* 2009).

The safety pharmacology studies are defined as those that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions, in relation to exposure in the therapeutic range. The respiratory system is an essential part of the core battery recommended in safety pharmacology studies (ICH 2000). The effects of the test substance on the respiratory system should be assessed appropriately. Clinical observations of animals are generally not adequate to assess respiratory function. Respiratory rate (RR) and other measures of respiratory function such as airway resistance, compliance, pulmonary arterial blood pressure, blood gases, and pH are recommended. Those

parameters should be quantified by using appropriate methodologies (ICH 2000).

This literature review will include the anatomy and physiology of the respiratory system of the different species, rats, dogs and monkeys used in this study. The rules and use of safety pharmacology will also be included.

LITTERATURE REVIEW

1. ANATOMY OF THE RESPIRATORY SYSTEM

The anatomy of the respiratory tract differs among species. The shape of both the upper and lower respiratory tract; the extent, shape, and pattern of the turbinate bones; the branching patterns of bronchi; the anatomy of terminal bronchioles, including collateral ventilation; the lobation and lobulation of the lungs; the thickness of the pleura; the completeness of the mediastinum; relationship of pulmonary arteries to bronchial arteries and bronchioles; the presence of vascular shunts and the blood supply could be different from one species to the other. Each variation in the anatomic structure could imply possible variations in the respiratory function (Reznick 1990).

However, some animal species have anatomically similar respiratory tracts. For instance, the dogs, monkeys and rats are similar while horses are closer to the human respiratory tract (Merck 2008). The respiratory system can be subdivided into the upper and the lower respiratory tracts based on anatomical features (Carter 2007).

1.1. The upper respiratory tract

The upper respiratory tract includes the nose, nasal passages, pharynx, larynx, and trachea (fig. 1), In addition to the obviously wide range of size and external shapes of the nose, there are also interspecies differences in the internal anatomy and physiology of the upper respiratory tract.

The upper respiratory structures of the tract act to tunnel fresh air down, from outside to inside the body. The upper airways are important because they must always stay open to be able to breath.

The nose assists in the production of sound and in olfaction. The most important function is to warm and to humidify the air through a highly vascularized mucus membrane covering the inside of the nose. The upper one-third of the nasal cavity is lined with olfactory epithelium which sits on the

lamina propria containing numerous serous and mucus glands while the lower two-thirds are lined with pseudostratified ciliated columnar epithelium containing high amount of goblet cells. Goblet cells, with microvilli on the surface, are present through the respiratory tract. Those cells are responsible for mucus secretion. The *microvilli* retain particles in the pharynx where they are swallowed to prevent foreign bodies to penetrate the lungs (Rusell 2004). The rat has a smaller percentage of goblet cells than man. Also, secretory cells are more frequent in rats compared to humans, except in the terminal bronchioles where they are comparable (Miller *et al.* 1993). In the resting animal, the nasal cavity, pharynx and larynx, provide approximately 60% of the frictional resistance to breathing (Cunningham & Klein 2007). In animals, nasal resistance can be decreased by dilation of the external nares and by vasoconstriction which reduces the volume of blood in the vascular sinuses within the nasal mucosa and, as a consequence, the mucosal thickness decreases and the space available for air within the nose increases(Cunningham & Klein 2007).

The pharynx extends from the base of the skull to the inferior border of the cricoid cartilage. It is divided into 3 parts: nasopharynx, oropharynx and laryngopharynx. The oropharynx also has a digestive function. It is delimited by the soft palate superiorly, the basis of the tongue inferiorly, the palatoglossal and the palatopharyngeal arches on lateral sides. The laryngopharynx or hypopharynx also has a digestive function and lies caudally to the larynx, extending from the superior border of the epiglottis and the pharyngo-epiglottic folds to the inferior border of the cricoid cartilage, when it narrows and becomes in continuity through the esophagus (Moore *et al*. 2006). Both are lined with non-keratinized stratified squamous epithelium. The nasopharynx includes the respiratory area, which is adapted to its main functions of filtering, warming, and humidifying (McGowan *et al.* 2003). It lies superior to the soft palate and is the posterior extension of the nasal cavities (Moore *et al.* 2006).

The larynx is located at the crossroads of the air and food passages. It includes the epiglottis, the thyroid, as well as the arytenoid and cricoid

cartilages. The larynx, by its position, prevents the accidental introduction of foreign bodies in the trachea. Its cranial end is attached to the hyoid bone and lies below the epiglottis, while its caudal end is in continuity with the trachea. One of the larynx main functions is to assist in warming and humidifying incoming air. The epiglottis is a structure, which serves to guide the larynx upwardly behind the soft palate so it can lock into the nasopharynx.

In rats, the larynx began at the opening bounded anteriorly by the free border of the epiglottis. The length of the larynx is 4–5 mm. The cricoid cartilage formed a complete ring at the inferior aspect of the larynx and is fixed to the tracheal rings (Nayci 2004). The thyroid cartilage, is a wedge shaped structure of which sides are called *laminae.* The thyroid cartilage articulates with the cricoid cartilage at the inferior side, cornually and bilaterally. The arytenoid cartilages are bilaterally situated and shaped like small three side pyramids, the bases of which are concave, representing the articular facets that glide upon the corresponding facets of the posterosuperior aspect of the cricoid lamina (Harvey 1993). The cricoid cartilage sits next to the thyroid; its function is to provide attachments for the various muscles, cartilages, and ligaments involved in opening and closing the airways and in vocalization production. The cricoid cartilage is roughly circular in shape inferiorly, and above, it follows the outline of the glottis. In other non-human mammals, cricothyroid joints vary in size and may be absent (Norris 1995). The principal role of airway smooth muscle is to control airway calibre, so the balance between resistance to airflow and physiological dead space is optimised (Matera *et al* 2002).

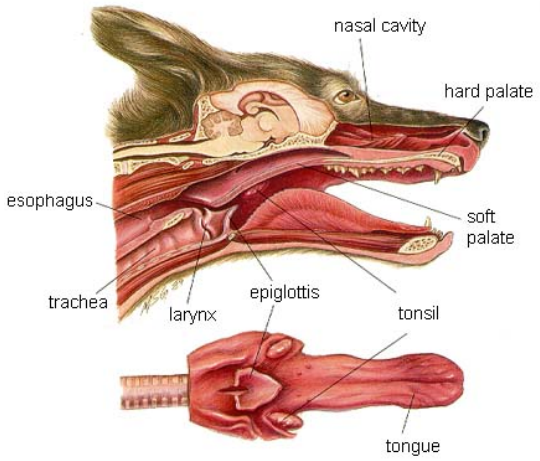

Figure 1. Schema of the upper respiratory tract of dogs (Printed from http://www.vetmed.wsu.edu/cliented/anatomy/dog_resp.aspx)

1.2. The lower respiratory tract

The lower respiratory tract includes the bronchi, the bronchioles, and the lungs. The lower respiratory tract structure varies both within a species and among specie. Irregular dichotomous and trichotomous airway branching patterns are present in human and nonhuman primate lungs. In contrast, the dog and common laboratory rodents exhibit a predominantly monopodial branching system (Miller *et al* 1993). In most laboratory rodents, the conducting airway terminates abruptly, therefore non-cartilaginous non-alveolarized airways, terminal brochioles open into a completely alveolarized airway (Bal & Ghoshal 1988). The trachea is nearly but not quite cylindrical, being flattened on the posterior. The trachea helps to direct inspired air to the gas exchanging regions of the lung (West 2005).

In dogs, the trachea extends from a transverse plane through the middle of the axis to a plane between the fourth and fifth thoracic vertebrae. It is composed of approximately 35 C-shaped tracheal cartilages. They are open dorsally and the space is bridged by the tracheal muscle (Evans *et al* 1988). At examination the trachea will be felt as a firm, tubular structure extending

throughout much of the cervical length on the vertical surface of the neck near the midline, the cartilaginous nature of the trachea allows it to be grasped between the fingers. Incomplete cartilaginous rings are bridged by the trachealis muscle (Smith 1999).

In rats, the length and internal diameter of the trachea is 25–27 mm and 3–4 mm, respectively. The cranial surface of the trachea is convex, and covered, in the neck area, in a cranio-caudal direction, by the isthmus of the thyroid gland, the inferior thyroid veins, the *arteria thyroidea ima,* the sternothyreoideus and sternohyoideus muscles, and the cervical fascia. Caudally, it is in contact with the esophagus. Laterally, in the neck, it is in relation with the common carotid arteries, the right and left lobes of the thyroid gland, the inferior thyroid arteries and, the recurrent nerves. In the thorax, it lies in the cranial mediastinum, and is in relation on the right side with the pleura and right vagus, and near the root of the neck, with the innominate artery. On its left side are the left recurrent nerve, the aortic arch, and the left common carotid and subclavian arteries (Gray 2000). The muscular tissue consists in two layers (longitudinal and transverse) of non-striated muscle. The longitudinal fibers are external, and consist of a few scattered bundles. The transverse fibers are internal and form a thin layer, which extends transversely between the ends of the cartilages (Goldbloom *et al.* 1960). The mediastinum in primates and dogs are comparable (Miller *et al.* 1996). This structure can be divided into a cranial part, lying cranial to the heart; a middle part, containing the heart; and a caudal part, lying caudal to the heart. The caudal mediastinum is thin. It attaches to the diaphragm far to the left of the median plane. Cranially it is continous with the middle mediastinum (Evans 1988).

In rodents, the potential space between the medial walls of the two visceral pleurae is called the mediastinum. It is nearly filled with structures including an endocrine gland called the thymus and the parietal pericardium (Wingerd 1988). The tracheobronchial tree is a branching system that delivers air to the alveoli. The number of branches depends on the animal's size. For instance, mice have about 10 and horses 40 or more. The tracheobronchial tree is

lined by a secretory, ciliated epithelium. The bronchi are supported by cartilage and supplied with bronchial glands and goblet cells, the secretions of which contribute to the mucous lining of the airways. The smaller airways, known as bronchioles, lack cartilages, glands and goblet cells. With the exception of the trachea and the cranial part of the mainstream bronchi, the airways are intrapulmonary. Alveolar septa attach to the outer layers of the airways so that tension within the septa pulls the airways open and helps to maintain their patency (Cunningham & Klein 2007).

The alveolar ducts and alveoli primarily consist of simple squamous epithelium that permits rapid diffusion of oxygen (O_2) and carbon dioxide (CO_2). Gas exchange between the air in the lungs and the blood in the capillaries occurs across the walls of the alveolar ducts and alveoli (Bray *et al.* 1999).

The alveolar duct branching system of the rat is complex (Miller *et al.* 1993). It is a three dimensions system in which multiple branches may occur within the distance of a single alveolus. The alveolar duct system of the rat is only about 100 μm.

In primates, the bronchioles are arranged stereotaxically like those of other mammalian lungs. The four bronchiole systems, dorsal, ventral, medial, and lateral, arise from both bronchi, respectively, although some bronchioles are lacking. In the right lung, the bronchioles form the upper, middle, accessory, and lower lobes, while in the left lung, the upper and accessory lobes are lacking and bi-lobed middle and lower lobes are formed (Nakakuki 1986).

The lungs are a pair of sponge-like organs located inside the thoracic cavity. They are not directly attached to the ribs. They are suspended by a double-walled sac called pleura (Wilmore *et al.* 2008). The inner layer of the sac (visceral pleura) tightly adheres to the lungs, and the outer layer (parietal pleura) is attached to the wall of the chest cavity. The two layers are separated by a thin space, called the pleural cavity, filled with pleural fluid allowing the inner and outer layers to slide over each other, and prevent them from being easily separated. The liquid allows the two pieces to slide over one another but makes

them difficult to separate. In the thorax, therefore, the pleural fluid mechanically links the lungs to the thorax so that the respiratory system behaves as a single unit. The lungs of most species have a total of six lobes, each supplied by a lobar bronchus, which gives rise to daughter bronchi. Even in specie such as the horse that lack lobation, the same pattern of six lobar bronchi is still present. At each division of a parent bronchus, the diameter of the other is similar to that of the parent (Cunningham & Klein 2007).

The primarily function of the lungs is to allow O_2 to move from the air into the venous blood and CO_2 to move out (West 2005). The air enters through the trachea going to the left and right bronchi and branch many times throughout the lungs, until they eventually form a little thin walled air sacs or bubbles, known as the alveoli. The alveoli are the sites of gas exchange with the blood (Widmaier *et al.* 2006).

The lungs present few differences between laboratory animal species. In dogs, each lung is roughly triangular and has apex, costal, medial, diaphragmatic surfaces, dorsal, ventral, and caudal borders. Each lung is divided by deep fissures into distinct lobes. The caudal fissures of the dog's lungs correspond to the oblique fissures of the human lungs (Ishaq 1980). Dogs like humans, have right and left lungs. Both sides of the lungs are further divided into lobes. Inside the lungs, the bronchi divide into smaller and smaller tubes, called bronchioles, much like branches of a tree divide into smaller and smaller branches.

In primates, the lungs of the rhesus monkeys are generally separated into six lobes. The left lung has two lobes, cranial and caudal, of which the cranial is further segmented into a cranial and caudal portion. The right lung has four distinct lobes in the rhesus monkeys. The lungs are lined by connective tissue band with a mesothelial surface facing the pleural space (Wolfe-Coote 2005).

In the rat, the lungs are large spongy structures. The right lung is divided into four lobes: cephalic, medial, caudal and postcaval (Fig. 2) (Wingerd 1988).

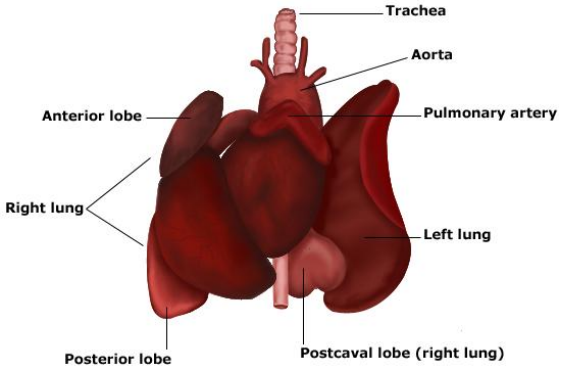

Figure 2. Rat lungs anatomy (Printed from www.tutorvista.com)

1.3. Pulmonary blood flow

The lung receives blood flow from two circulatory systems: the pulmonary circulation and the bronchial circulation. The pulmonary circulation receives the total output of the right ventricle, perfuses the alveolar capillaries and participates in gas exchange. The bronchial circulation, a branch of the systemic circulation, provides a nutritional blood supply to airways and other structures within the lung (Cunningham & Klein 2007).

The pulmonary circulation carries roughly the same flow as the systemic circulation. The arterial pressure and the vascular resistance are normally only one-sixth as great. The media of the pulmonary arteries is about half as thick as in systemic arteries of corresponding size. In the larger vessels, it mainly consists of elastic tissue but in the smaller vessels, it is mainly muscular. The transition in vessels is close to 1 mm diameter. Pulmonary arteries lie close to the corresponding air passages in connective tissue sheaths (Lumb 2006). The bronchial circulation provides nutrient blood flow to airways, large blood vessels, and the pleura (Mc Laughlin *et al.* 1961).

The pulmonary circulation begins at the main pulmonary artery, which receives the mixed venous blood pumped by the right ventricle. The main pulmonary arteries that accompany the bronchi are elastic, but the smaller arteries adjacent to the bronchioles and the alveolar ducts are muscular

(Cunningham & Klein 2007). This, then branches successively like the system of airways and, indeed, the pulmonary arteries accompany the airways as far as the terminal bronchioles. Beyond that, they break up to supply the capillary bed which lays in the alveoli walls. The pulmonary capillaries form a dense network in the alveolar wall which makes an exceedingly efficient arrangement for gas exchange (Fig. 3). The oxygenated blood is then collected from the capillary bed by the small pulmonary veins that run between the lobules which drain into the left atrium (West 2005).

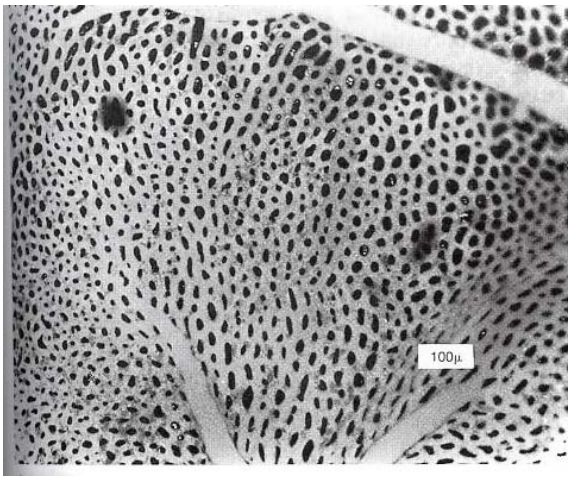

Figure 3. Surface view of capillaries in alveolar wall(Printed from: Guyton, A.C. & Hall, J.E., 2006. Textbook of Medical Physiology., p 497)

The amount of smooth muscle in the media of pulmonary arteries determines the reactivity of the vasculature. The small pulmonary arteries lead into pulmonary capillaries, which form an extensive branching network of vessels within the alveolar septum, covering almost the alveolar surface. Not all capillaries are perfused in the resting animal. As a result, vessels that are unperfused can be recruited when pulmonary blood flow increases. Pulmonary veins with thin walls conduct blood from capillaries to the left atrium and also form a reservoir of blood for the left ventricle (Fig 4) (Cunningham & Klein 2007).

In the bronchioles, oxygenated arterial blood flows to the lungs through small bronchial arteries that originate from the systemic circulation. After this

bronchial and arterial blood has passed through the supporting tissues, it empties into the pulmonary veins and enters the left atrium. Therefore, the flow into the left atrium and the left ventricular output are about 1 to 2 per cent greater than the right ventricular output (Guyton *et al.* 2006).

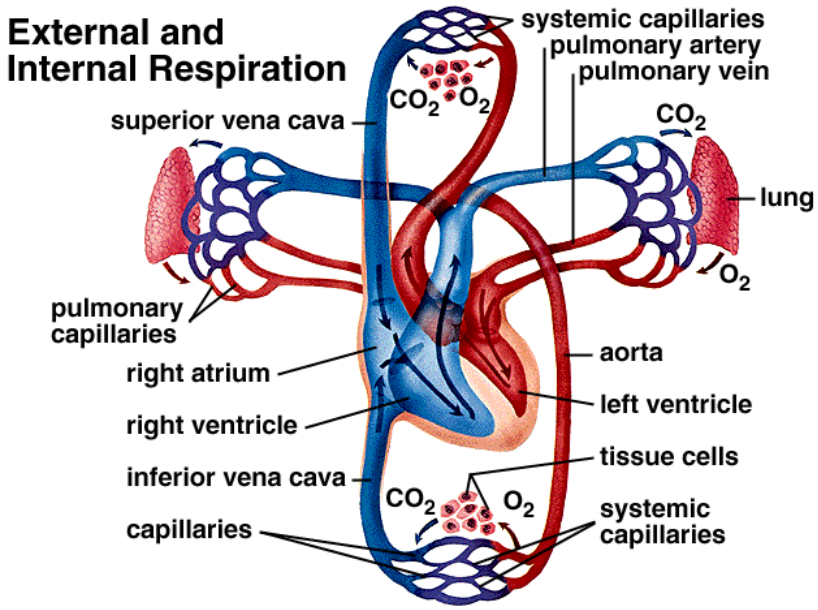

Figure 4. Pulmonary blood circulation (Printed from www.mhhe.com)

1.4. Muscles of respiration

Energy provided by muscles causes air to enter the lungs during inhalation. During exhalation, much of the energy causing air to leave the lungs is provided by the elastic energy stored in the stretched lung and the thorax (Cunningham and Klein 2007). There are several muscles which play an important role in the inspiration and expiration process during breathing (Adams 1998).

The inspiration muscles are composed of the diaphragm, the external intercostal muscles, and the accessory muscles. The accessory muscles include the scalene muscles and the sternomastoids. Other muscles playing a minor role include the *alae nasi*, which causes flaring of the nostrils and, small muscles in

the neck and head. The primary inspiration muscle is the diaphragm, which is a domed musculotendinous sheet separating the abdomen and thorax and innervated by the phrenic nerve, responsible for an active inhalation. The external intercostals muscles also are active during inhalation. The fibers of these muscle contraction moves the ribs rostrally and outward. The relative contributions of diaphragmatic and costal movements to ventilation under different metabolic demands are not well defined in animals. Because the cranial ribs support the forelimbs in quadrupeds, they participate less in ventilation than do the more caudal ribs. Other inspiratory muscles include those connecting the sternum and head. These muscles contract during stenous breathing and move the sternum rostrally (Cunningham & Klein 2007).

During expiration, the most important muscles are those of the abdominal wall, including the *rectus abdominis*, internal and external oblique muscles and *transversus abdominis*. The internal intercostal muscles also assist during the process (West 2005). Contraction of the abdominal muscles increases abdominal pressure, which forces the relaxed diaphragm forward and reduces the size of the thorax. The fibers of the internal intercostals are directed cranio-ventrally, from the cranial border of one rib to the caudal border of the next cranial rib, so their contraction decreases the size of the thorax by moving the ribs caudally and ventrally. As the thorax becomes smaller, the thoracic pressure increases and forces air out of the lungs (Cunningham & Klein 2007).

2. PHYSIOLOGY OF THE RESPIRATORY SYSTEM

In mammals, the function of the respiratory system is essential to maintain homeostasis (National Research Council 2003). Its major function is gas exchange between the external environment and the organism's circulatory system. This exchange facilitates oxygenation of the blood with concomitant removal of CO_2 and other gaseous metabolic wastes from the circulation as water, sulfates, phosphates and nitrogen, (Widmaier *et al.* 2006).

The role of the inspiration and expiration is essential for breathing helping to change the intrathoracic pressure involving different muscles and to permit the exchange of O_2 and CO_2 (Schwartzstein & Parker, 2005).

2.1. Inspiration process

Muscular activity is involved in the inspiration process. Various muscles are involved, the diaphragm being the main one. Those muscles act to increase the thoracic volume, causing intra-pleural and alveolar pressure to fall creating an alveolar-mouth pressure gradient, drawing air into the lungs.

Resting animals breathe slowly and have low flow rates. In this situation, the primary work of the respiratory muscles is against the compliance of the lungs (Cunningham & Klein 2007). However, during exercise or in response to a stressful situation, they may contract vigorously (West 2005) and respiratory muscle activity increases in order to generate an increase in minute ventilation (MV). In cursorial (running) mammals, ventilation is synchronized at canter and gallop, but not at the trot. Inhalation occurs as the forelimbs are extended and the hind limbs are accelerating the animal forward. Exhalation occurs when the forelimbs are in contact with the ground. In the galloping horse and perhaps in other galloping quadrupeds, much of the increase in size of the thorax during inhalation is a consequence of elongation of the trunk rather than an increase in the diameter of the thorax (Cunningham & Klein 2007).

2.2. Expiration process

Expiration is present at the end of inspiration. Expiration in a resting animal is a passive process that does not require muscle contractions (except in horses, where expiration is a passive – active process). Relaxation of the muscles contracted during inspiration permits the intrinsic elastic properties of the lungs and the thoracic wall to recoil to the original volume. The return to the original volume increases the intra-pulmonary pressure so it is greater than atmospheric, and air is forced out of the lungs. Forced expiration is an active process that

forces more air from the lungs that would occur during normal passive expiration. Forced expiration requires contraction of abdominal muscles to force viscera against the diaphragm and contraction of other muscles to pull the ribs caudad. Both of these actions reduce the size of the thoracic cavity and permit recoil of the lungs to a smaller volume than typical for resting expiration (Frandson *et al.* 2003).

At the end of a normal exhalation, some air remains in the lungs. This air volume is known as functional residual capacity (FRC). At FRC, the pressure in the pleural cavity (Ppl) surrounding the lung is approximately 5 cm H2O below atmospheric pressure. Ppl decreases during inhalation as the thorax enlarges and the respiratory muscles perfom work to stretch the elastic lung. Resting animals breathe slowly and have low flow rates. In this situation the primary work of the respiratory muscles is against the compliance of the lungs. When the respiratory rate increases (*e.g.* during exercise), flow rates increase, and more energy is used to generate flow against the frictional resistance of the airways (Cunningham & Klein 2007).

2.3. Gas exchange in the lungs

The lung is the organ of gas exchange, providing the means of transferring O_2 from the air to the blood for subsequent distribution to the tissues. At the same time, it enables CO_2 removal from the blood which is then exhaled to the atmosphere. Gas exchange occurs by passive diffusion for CO_2 but for O_2, haemoglobin (Hb)-induced attraction helps a lot (Warrell *et al.* 2005). At rest, 100 mm Hg pressure of O_2 in the alveoli exceeds by about 60 mm Hg the 40 mm Hg O_2 pressure in blood that enters the pulmonary capillaries. Consequently, O_2 dissolves and diffuses through the alveolar membranes into the blood (McArdle *et al.* 2006). Oxygen is poorly soluble in water and therefore in plasma. Because of this low solubility, most animals need an oxygen carrying pigment to transport sufficient O_2 to the tissues. When blood in the pulmonary capillaries flows past the alveoli, O_2 diffuses from the alveoli into the blood until the partial

pressures equilibrate, and there is no further driving pressure difference. Without Hb, which transports the majority of the O_2, the cardiac output would have to be inordinately high to maintain the O_2 supply to the body organs (Cunningham & Klein 2007). In contrast, CO_2 exists under a slightly greater pressure in returning venous blood than in the alveoli causing a net diffusion of CO_2 from the blood into the lungs. Gas exchange occurs so rapidly in the healthy lung that alveolar-gas / blood gas equilibrium takes place in about 0.25 second or within one third of blood transit time through the lungs. Even in high intensity exercise, red blood cell velocity through a pulmonary capillary generally does not exceed by more than 50% its velocity at rest (McArdle *et al.* 2006).

2.4. Blood flow and O_2 transport

Systemic arterial blood enters capillaries throughout the body; it is separated from the interstitial fluid by only the thin capillary wall which is highly permeable to both O_2 and CO_2. The interstitial fluid, in turn, is separated from the intracellular fluid by the plasma membranes of the cells which are also quite permeable to O_2 and CO_2. The supply of the new O_2 to the alveoli and the consumption of O_2 in the cells create partial pressure in O_2 (PO_2) gradients that produce net diffusion of O_2 from alveoli to blood in the lungs and from blood to cells in the rest of the body (Widmaier *et al.* 2006).

In the blood circulation, O_2 is present in two forms: dissolved in the plasma and red blood cells (RBC) cytoplasm and combined to Hb molecules in the RBC (Wnek and Bowlin, 2008). The amount of O_2 dissolved in blood is directly proportional to blood PO_2. Relatively insoluble in water, only 3 ml of O_2 can be dissolved in 1 L of blood at normal arterial PO_2 (100 mm Hg) (Widmaier *et al.* 2006). The pulmonary capillary blood equilibrates with the alveolar O_2 tension of 100 mm Hg, therefore, 0.3 ml of O_2 dissolves in each decilitre of blood. If an animal breathes pure oxygen, the alveolar oxygen tension increases to approximately 600 mg Hg, and 1.8 mL of oxygen dissolves in each decilitre of plasma (Cunningham & Klein 2007).

Oxygen transport is the volume of O_2 moved through the circulation in a unit of time. Cardiac output or blood flow to a specific site is important to O_2 transport and tissue delivery. Blood O_2 content depends on the pulmonary capillary PO_2 produced by gas exchange, the amount of Hb present, and the slope of O_2-Hb dissociation curve (Stein, 1998). The binding of O_2 is a four-step process, and the O_2 affinity of a particular haem is influenced by the oxygenation of the others. This means that when the first haem unit is oxygenated, O_2 affinity of the second haem unit is increased and so on. These haem-haem interactions are responsible for the sigmoid shape of the oxy-Hb dissociation curve (Cunningham & Klein 2007).

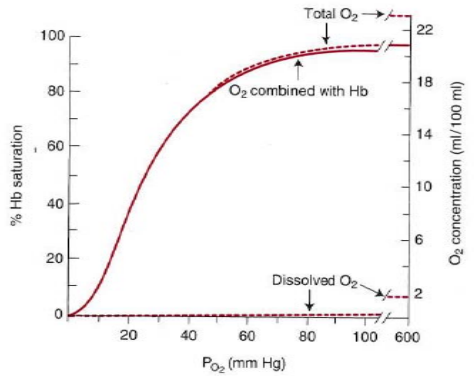

Figure 5. O_2 dissociation curve. (Printed fromo: West, J.B., 2005. Respiratory Physiology: The Essentials. 7th ed. Lippincott Williams & Wilkins., p76)

2.5. Carbon dioxide (CO_2) transport

During steady-state conditions, approximately 80 molecules of CO_2 are exhaled from the lungs for every 100 molecules of O_2 taken up from the alveoli into pulmonary capillary blood (148-150 molecules). The CO_2 is produced in the tissues and carried to the lungs through the venous blood in three forms: dissolved CO_2, a chemical combination of CO_2 and Hb and as bicarbonate

(HCO_3^-) which is the major form (90%) (Costanzo 2006). When considering tissue CO_2 content, the difference between CO_2 and O_2 amounts may be as great as tenfold because CO_2 is 23 times more soluble than O_2 in plasma (Brown *et al* 2006). The total atmospheric pressure is equal to 760 mm Hg; CO_2 is almost absent in the atmospheric air, this explains the effective diffusion gradient for CO_2 from the body to the air (Reece 2004).

At a normal arterial PCO_2 of 40 mm Hg, the amount of CO_2 transported in solution is equal to 28 mL/L blood or equivalent to 100 mm Hg PO_2. Because of its higher solubility, approximately 6% of total blood CO_2 is in physical solution compared to 1.5 % of O_2 dissolved in arterial blood. The concentration of dissolved CO_2 is greater in venous blood than in arterial blood. Rapidly CO_2 diffuses out of the cell due to its higher intracellular *versus* blood partial pressure . Once CO_2 diffuses out of the cell, it quickly moves into the RBC. Once inside RBC, CO_2 is chemically converted in the presence of water by the enzyme carbonic anhydrase to carbonic acid ($H_2CO_3^-$) and finally into a hydrogen ion (H^+) and HCO_3^- (Brown *et al.* 2006).

2.6. Respiratory receptors

Lungs contain sensory receptors that communicate with the respiratory centers through vagal nerve afferents. At rest, the chemical state of the blood exerts the greatest control on pulmonary ventilation. Variations in arterial PCO_2, pH, PO_2, and temperature activate sensitive neural units in the medulla and arterial system to adjust ventilation and maintain arterial blood chemistry within narrow limits (McArdle *et al.* 2006).

Different patterns during the respiratory process need to be coordinated in order to avoid abnormal breathing patterns (Mc Ardle *et al.* 2006). Some of abnormal breathing patterns are tachypnea, bradypnea, apnea, and hyperapnea (Springhouse, 2008). Many types of receptors are involved in the respiratory control: chemoreceptors (central and peripheral), lung receptors (irritant,

pulmonary stretch, and juxtapulmonary), receptors in the chest wall, several receptors in the trachea, laryngeal, arterial baroreceptors, and pain receptors.

The chemoreceptors monitor blood gas tension in arterial $PaCO_2$, PaO_2 and pH, and helps keep MV appropriate to metabolic demands of the body. The chemoreceptors respond to hypercapnia (↑ CO_2), hypoxia (reduction of O_2), anoxia (absence of O_2) and acidosis (↓ HCO_3^- or ↑ PCO_2). The most sensitive system is the one responding to $PaCO_2$. The central chemoreceptors are tonically active and vital for respiration maintenance. Eighty percent of ventilation drive is a result of central chemoreceptors stimulation. When they are inactivated, the respiration ceases. These receptors are located in the brainstem on the ventrolateral surface of the medulla, close to the exit of cranial nerves IX and X. They are anatomically separated from the medullary respiratory control center. These chemoreceptors respond to H^+ concentrations. A rise in $[H^+]$ increases ventilation, while a lowering decreases it (Bijlani 2004). The peripheral chemoreceptors are located around the carotid sinus and aortic arch. Stimulation of peripheral chemoreceptors has both cardiovascular and respiratory effects. Peripheral chemoreceptors provide the primary site to detect arterial hypoxia and by reflex initiate a ventilatory response (McArdle *et al.* 2006).

The carotid bodies are chemoreceptors sensitive to CO_2 and O_2 concentrations in the blood (Unchoa & Cameiro, 2005). They are located close to the bifurcation of the internal and external carotid arteries. The latter appear to be most active in the foetus and of little importance in the adult. The carotid bodies are small, pink nodular structures with extremely high blood flow per gram. This high blood flow and metabolism ratio allows the carotid bodies to obtain their O_2 needs from dissolved O_2. Carotid bodies contain several cell types. Type I cells, or glomus cells, synapse with afferent nerves that transmit information back to the brain. These glomus contain a variety of neurotransmitters, including catecholamines, especially dopamine. Glomus cells are probably responsible for the chemosensitivity of the afferent nerve terminals. Type II cells support the axons and blood vessels that ramify within the carotid

body. When the carotid bodies are perfused with blood that has a low PaO_2, high $PaCO_2$, or low pH, firing rates in the carotid sinus nerve afferent fibers increase. As $PaCO_2$ increases and pH decreases, there is an almost linear increase in ventilation, the response to PaO_2 is non linear (Cunningham & Klein 2007). Peripheral chemoreceptors are sensitive to PaO_2, $PaCO_2$, pH, blood flow, and temperature (McGowan *et al.* 2003). The activity in the nerve fibers is directly related to the degree of hypoxia at the chemosensory cells (Kline *et al*. 2005).

The carotid bodies respond to the autonomic nervous system (Koyama *et al.* 2000). The sympathetic action vasoconstricts and increases sensitivity to hypoxia while the parasympathetic action vasodilates and reduces sensitivity to hypoxia. Unlike central chemoreceptors, peripheral chemoreceptors are directly stimulated by blood pH (McGowan *et al.* 2003). The vagus contains myelinated afferents slowly adapting pulmonary stretch receptors which are stimulated by changes in lungs volumes. Stretch depolarizes these receptors, sending action potentials to respiratory centers in the brain *via* the vagal nerves (Johnson & Byrne, 2003). The stretch receptors are situated in the smooth muscle of the bronchial walls and respond to changes in transmural pressure (McGowan *et al.* 2003). When these areas are excessively stretched, the information is relayed to the expiratory center. The center responds by shortening the duration of the inspiration, which decreases the risk of overinflating the respiratory structures. This response is known as Henri-Breuer reflex (Wilmore *et al.* 2008).

The respiratory receptors do not act alone in controlling breathing. Breathing is also regulated and modified by the changing chemical environment of the body. For example, sensitive areas in the brain respond to changes in CO_2 and H^+ levels. Central chemoreceptors are stimulated by an increase of H^+ ions in the cerebrospinal fluid. The goal of respiration is to maintain appropriate levels of blood and tissue gases and to maintain proper pH for normal cellular function. Small changes in any of these, if not carefully controlled, could impair physical activity and jeopardize health (Wilmore *et al.* 2008).

During exercise, resting arterial Hb saturation in O_2 is close to 100% in normal conditions (no pathology to Hb or RBC) and O_2 content cannot be raised significantly. Oxygen delivery to exercising muscle is augmented by increasing muscle blood flow, made possible by metabolic vasodilatation. Oxygen extraction from the delivered blood is increased. Mixed venous blood has reduced PvO_2 and increased $PvCO_2$. Passive limb moving stimulates ventilation in both anaesthetized and awaken animals. This is a reflex due to receptors presumably located in joints or muscles. It may be responsible for the abrupt increase in ventilation that occurs during the first seconds of exercise (West 2007).

At high altitude, barometric pressure falls progressively. For instance, it falls from around 101 kPa (760 mmHg) at sea level to 33.6 kPa (252 mmHg) on the summit of Everest; however, O_2 fraction remains constant at 0.209. If ventilation remains unchanged, reduced inspired PO_2 inevitably leads to reduced PaO_2 but $PaCO_2$ will be unaltered (Ward *et al.* 2006). At such altitude, metabolic rate is unchanged in resting animals. Therefore increased ventilation causes a decrease in $PaCO_2$. This decrease in $PaCO_2$ inhibits ventilatory drive, which limits the hypoxic ventilator response by the carotid body chemoreceptors (Johnson *et al.* 2003). The RBC concentration increases and the oxyHb dissociation curve is shifted to the right. However, at very high altitude the very low $PaCO_2$ shifts the curve to the left and the beneficial effect of increased O_2 binding in the lungs seems to outweigh the impaired O_2 release in the tissues. The widespread hypoxia vasoconstriction in the lungs is helpful (Ward *et al.* 2006). Hypoxic vasoconstriction is unique to pulmonary circulation. The pulmonary response is part of a self-regulatory mechanism by which pulmonary capillary blood flow is automatically adjusted to alveolar ventilation for maintaining the optimal balance of ventilation and perfusion (Dumas *et al.* 1999). It increases pulmonary vascular resistance and those living at high altitude may develop right ventricular strain and failure (Ward *et al.* 2006). The

pulmonary vascular resistance may persist through childhood and adult life if the individual remains at high altitude (Rudolph 2001).

2.7. Respiratory mechanics

Respiratory mechanics explains how the forces generated by respiratory muscles cause effective ventilation of the alveoli. Resistance and compliance are two physical factors important in determining the relationship between pressure and flow in the lungs, and in the distribution of ventilation to different regions of the lungs. The effectiveness of ventilation not only depends on the amount of fresh air reaching the alveoli, but also on the matching of ventilation to blood flow in different regions of the lung (Johnson *et al.* 2003).

2.7.1. Resistance

Respiratory resistance is a combination of resistance to gas flow in the airways and resistance to deformation of tissues of both the lungs and the chest wall. In normal lungs, respiratory resistance is controlled by changes in airway diameter, mainly in small airways and bronchioles. Gas flows from a region of high pressure to one of lower pressure. The rate at which it does so is a function of the pressure difference and the resistance to gas flow. In the healthy subjects, the small airways only make a small contribution to total airway resistance because their aggregate cross-sectional area increases to very large values after about eight generations. Gas flow velocity and airway diameter decrease in successive airway generations. The airway lining will influence frictional resistance more with turbulent than with laminar flow, with changes in mucus consistency that occur in many airway diseases (Lumb 2006). The factors determining airway resistance are analogous to those determining vascular resistance in the circulatory system. Those factors include tube length, tube radius, and interactions between moving gas molecules. Physical, neural and chemical factors affect airway radii and therefore resistance. One important physical factor is the trans-pulmonary pressure which exerts a distending force on the airways, just as on the alveoli. This is a major factor keeping the smaller

airways, those without cartilage, to support them from collapsing. Trans-pulmonary pressure increases during inspiration, airway radius becomes larger and airway resistance lower as the lungs expand during inspiration. The opposite occurs during expiration (Widmaier *et al*. 2006).

2.7.2. Compliance

Lung compliance is defined as the change in lung volume per unit change in trans-mural pressure gradient. There are two major determinants of lung compliance; first, the stretchability of lung tissues (particularly their elastic connective tissues, thus, a thickening of the lung tissues decreases lung compliance) and the other is the surface tension at the air-water interfaces within the alveoli. The surface of the alveolar cells is moist, so the alveoli can be pictured as air-filled sacs lined with water. At an air-water interface, the attractive forces between the water molecules, known as surface tension, make the water lining like a stretched balloon that constantly tries to shrink and resists further stretching. Thus, expansion of the lung requires energy not only to stretch the connective tissue of the lung, but also to overcome the surface tension of the water layer lining the alveoli (Widmaier *et al.* 2006). Compliance is usually expressed in L or mL per KPa or cmH_2O. The normal value of compliance is approximately 1.5 $L.kPa^{-1}$ or 150 $mL.cmH_2O^{-1}$. When an increase of peribronchial and perivascular fluid and thickening of the lung tissues occurs for instance, a decrease in lung compliance is observed (Lumb 2006), a condition called as stiff lungs.

Elastic recoil is expressed as elastance, which is the reciprocal of compliance. Only a quarter to a third of the lungs elastic recoil is caused by the elastic fibres of its alveolar walls, most is caused by surface tension at the alveolar tissue (Bray *et al.* 1999). Respiratory system elastance is known to increase with frequency breathing, and tissue resistance is known to decay hyperbolically over the range of physiological breathing frequencies, whereas

airway resistance remains fairly constant both in healthy and mildly constricted lungs (Lutchen *et al.* 1994).

2.7.3. Ventilation and Lung Mechanics

Breathing is caused by rhythmic contractions of skeletal muscles that are entirely dependent upon intact nervous connections from the medulla through the spinal cord, the phrenic and intercostal nerves. Breathing is thus the product of chemical and neural influences on a network of neurones, motor nerves, and muscles; the result being dependent on the mechanical properties of the chest, the lungs, and the airways (Whipp 1987). The act of breathing is performed against several impediments: elastic, resistive, visco-elastic, plasto-elastic, inertial and gravitational forces, compressibility of intra-thoracic gas, and distortion of the chest wall (Polese *et al.* 2005).

Ventilation is defined as the exchange of air between the atmosphere and alveoli. Like blood, air moves by bulk flow, from a region of high pressure to one of low pressure. In the respiratory system, pressures are expressed in relation to atmospheric pressure, which is 760 mmHg at sea level (West 2008). During ventilation, air moves into and out of the lungs because the alveolar pressure is alternately less than and greater than atmospheric pressure (Widmaier *et al.* 2006).

The intrapleural pressure causes the lung and thoracic wall to move in and out together during normal breathing (Widmaier *et al.* 2006). When the thorax expands and pull the lungs, they must comply and expand. The air must comply and smoothly flow to the alveoli. If the thorax and lungs are stiff or the airways are narrow, so the air does not move in and out of the lungs freely, pulmonary ventilation will be impaired (Sabyasachi 2007).

2.7.4. Elastic properties of the lungs

The elastic properties of the lung and of the chest wall to airflow resistance are part of the mechanic of breathing. The lung is a volume-elastic

container that tends to deflate itself. The deflation force exerted by the lungs is call elastic recoil pressure. This pressure increases with lung volume and the pressure requires maintaining inflation equals to the elastic recoil. Lung expansion is maintained either by negative pleural pressure at the outer surface of the lung or by positive pressure applied by the airway opening to the inner surface of the lung (Shields *et al.* 2004).

The movements of the lungs are entirely passive and result from forces external to the lungs. The pattern of lung response is governed by the physical impedance of the respiratory system. The most important impedances are: elastic resistance of lung tissue and chest wall, resistance from surface forces at the alveolar area, frictional resistance to gas flow through the airways, frictional resistance from deformation of thoracic tissues, and inertia associated with movement of gas and tissue. The surface forces have a predominant influence on the elastic properties of the lung at volumes higher than functional residual capacity, which implicates alveolar size as the major determinant of specific pulmonary elastance for a constant number of alveoli (Haber *et al.* 1983).

2.7.5. Pulmonary Volumes and Capacities

The volume of gas in the lungs can be divided into the following components:TLC, VC, IC, ERV, IRV, TV, RV and FRC (Fig 5). A capacity is defined as the summation of two, or more, volumes. From these variables, the most commonly used to test respiratory function are TV (amount of air breathed in or out during a quiet breathing), RV, VC and FRC. Those can be useful in diagnosing certain pulmonary diseases (Johnson et *al* 2003). Pulmonary volumes are either associated with the amount of air within them at any time or with the amount associated with breath (Reece 2004).

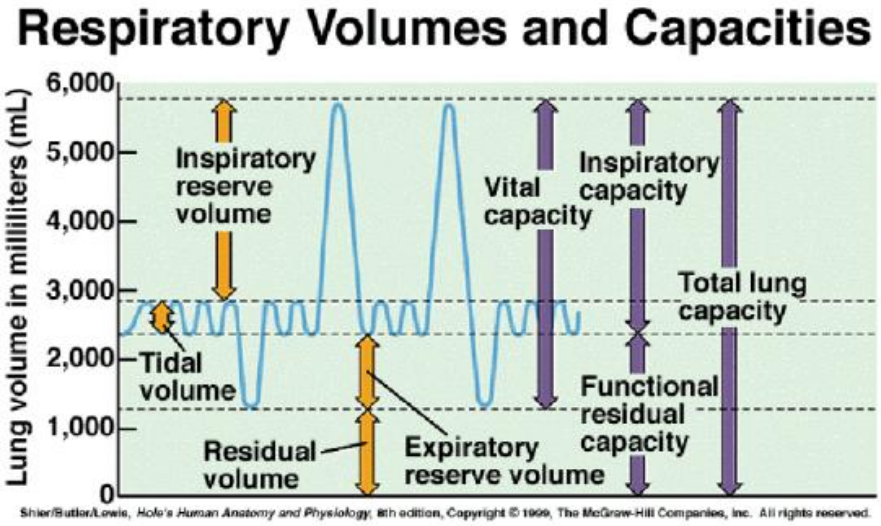

Figure 6. Respiratory volumes and capacities. Printed from (http://images.google.ca/imgres?imgurl=http://faculty.stcc.edu)

The O_2 needs of metabolism require that an animal take a certain volume of air into its lungs, essentially its alveoli, each minute. The total volume of air breathed per minute or MV (minute ventilation), is determined by the volume of each breath, known as the tidal volume (TV), and the number of breaths per minute, know as respiratory frequency or RR (respiratory rate).

$MV = TV \times RR$

The increase in MV, which must occur when an increase in metabolic rate demands more oxygen, can be brougth about through an increase in TV, RR or both. The IRV is the amount of air that can still be inspired after inhaling the TV, and ERV is the amount of air that can still be expired after exhaling the TV (Johnson *et al.* 2003). The RV is the amount of air remaining in the lungs after the most forceful expiration. The TLC is the sum of all volumes while VC is the sum of all volumes over and above the RV, which is also the maximum amount of air that can be inspired after the most forceful expiration; IC is the sum of TV and IRV, and FRC is the sum of ERV and RV (Reece 2004). Normal lung volumes provide lessons about respiratory physiology in healthy individuals.

The TLC is more than ten times larger than the normal TV, so there is a tremendous reserve capacity for increased ventilation with increased O_2 demand or reduced supply. This is part of the reason why pulmonary gas exchange is usually not a limiting factor in O_2 uptake at sea level in anyone except highly trained elite athletes (Johnson *et al.* 2003). Finally, FRC is the volume of air present in the lungs at the end of passive expiration (Sabyasachi 2007). A lowered or elevated FRC helps to diagnose some forms of respiratory disease.

2.7.6. Dead space

The anatomic dead space is the volume of the upper respiratory system where gas exchange do not occur. Dead space (Vs) can also be present within the alveoli. This alveolar dead space is caused by alveoli that are poorly perfused with blood, so that gas exchange cannot occur optimally. Physiological dead space is the sum of the anatomic and the alveolar dead space (Cunningham & Klein 2007). The strict definition of the anatomical dead space is the volume of an inspired breath which has not mixed with the gas in the alveoli. Because gas exchange effectively only takes place in the alveoli there is no CO_2 excreted into the dead space (Davies & Moore 2003). Essentially, all the gas exchanges between air and blood take place at the alveolar surface. At the end of the inspiration, the content of the alveoli has been diluted by inspired room air, which now also fills the anatomical dead space. At the end of expiration, the anatomical dead space is filled with alveolar air, and this partly used air is inhaled first in the next inspiration (Fig. 6) (Davies & Moore 2003). Not all of the air entering the respiratory system actually reaches the alveoli and takes part in gas exchange.

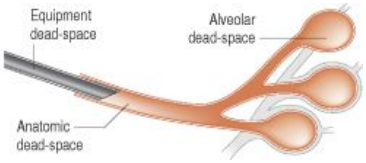

Figure 7. Anatomic and alveolar dead space
(Printed from http://img.tfd.com/vet/thumbs/gr356.jpg)

The structures included in the dead space are: nose and mouth, pharynx, larynx, trachea, bronchi and bronchioles, down to and including the terminal bronchioles. The fraction of each breath ventilating the dead space is known as the VD/TV ratio. The VD/TV varies considerably among species. In smaller species, such as dogs, it approximates 33%, whereas in some larger species, such as cattle and horses, it approximates 50% to 75%. Because the volume of the anatomic dead space is relatively constant, changes in TV, RR or both can alter the relative amounts of air that ventilate the alveoli and the dead space. These changes in TV and RR occur in animals during exercise and thermoregulation. For example, the small TV and high RR characteristic of panting in dogs cause more air to ventilate the dead space in order to cause water evaporation and heat loss. Cattle, pigs and mules subjected to heat stress also increase RR and dead space ventilation when trying to lose heat. In contrast to the effects of heat stress, cold stressed animals have a higher metabolic rate, which is necessary to maintain body temperature in cold conditions. This leads to an increase in both O_2 consumption and CO_2 production, making it necessary for the animal to increase alveolar ventilation and decrease dead space ventilation. Reducing the RR and increasing TV accomplish the latter adaptations (Cunningham & Klein 2007).

Anatomical dead space can be measured using Fowler's method which is based on the single breath nitrogen test (McGowan *et al*. 2003). This test measures inequalities of ventilation, useful to evaluate ventilatory capacity (West 2007). If some regions of the lung expand before others in the process of inspiration, they will receive inappropriately large parts of this dead space, and the regions receiving air later in inspiration will receive more fresh air. This type of dead space is called anatomical dead space because it measures the anatomical volume of the conducting airways. If each acinus or end respiratory unit was perfect, the amount of air received by each alveolus would be matched by the flow of blood through the pulmonary capillaries. In a normal healthy

person, anatomical and physiological dead space are almost equal, alveolar dead space being very small (<5 mL). However, the volume of alveolar dead space increases when lung disease alters ventilation / perfusion matching,. Physiological dead space is calculated, using the Bohr Equation (McGowan *et al* 2003). The Bohr Equation permits the determination of the sum of the anatomic and the alveolar dead space. It evaluates the measurable volume of CO_2 found in the mixed expired gas coming from the alveoli. The PCO_2 of the collected mixed expired gas can be determined with a CO_2 meter. The CO_2 meter is also often used to estimate the alveolar PCO_2 by analyzing the gas expelled at the end of a normal tidal expiration, the end tidal CO_2 (Levitzky 2007).

3. SAFETY PHARMACOLOGY

Safety Pharmacology is the discipline trying to predict whether a drug, if administered to human (or animal) population, is likely to be found safe (Pugsley *et al.* 2008). It is, with the primary pharmacodynamic studies which are those evaluating the mode of action and/or effects of a substance in relation to its desired therapeutic target and secondary pharmacodynamic studies which evaluate the mode of action and/or effects of a substance not related to its desired therapeutic, a part of the pharmacology discipline (FDA 1997).

The origins of Safety Pharmacology are grounded upon observations that organ functions can be toxicological targets in humans but the effects cannot be readily detected by standard toxicological testing (Mortin *et al.* 1997; Williams 1990; Zbinden, 1984). The objectives of safety pharmacology are: 1) to identify undesirable pharmacodynamic properties of a substance that may have relevance to its human safety; 2) to evaluate adverse pharmacodynamic and/or pathophysiological effects of a substance observed in toxicology and/or clinical studies; and 3) to investigate the mechanism of the adverse pharmacodynamic effects observed and/or suspected (ICH 2001).

The term first appeared in drafts of the ICH in 1997 (Bass *et al* 2004). Following that, in 1999, an Expert Working Group was formed and began their work to define the guidelines of Safety Pharmacology. A harmonized Safety Pharmacology guideline was finalized and adopted by the regional regulatory authorities over 2000–2001 (ICH 2001). They describe their implications in studies of the three vital systems: central nervous, cardiovascular and respiratory systems (Porslot *et al.* 2002).

Those guidelines were accepted in United States, Europe and Japan. Prior to 1990's, toxicological testing done on lead compounds was limited and did not allow to identify all adverse effects produced by those compounds. The U.S. and European regulations provided only general references to the evaluations of drug effects (Kinter *et al* 1994; Lumley, 1994).

Organ function assessments were inconsistent and often viewed as unimportant (Green, 1995; Proakis, 1994). In fact, during that period, it was discovered that some of those compounds showed rare and potentially lethal adverse effects later in the testing process (Pugsley *et al*. 2008).

The organ systems and functions most frequently responsible in these events were the cardiovascular, central nervous and renal systems, The result was almost always a critical care emergency (Kinter *et al* 1994). Such episodes contributed to the development of Safety Pharmacology.

The ICH guidelines have brought uniformity to the evaluations of new drugs for effects on organ functions (Bass *et al.* 2004). Within a short period, Safety Pharmacology had evolved into a discipline designed to bridge the gap between preclinical toxicology and preclinical and clinical drug development (Bass *et al.* 2004). However the creation of Safety Pharmacology has not resolved all problems with respect to detection of rare and lethal adverse effect liability and more problems have to be solved in order to quantify, with high certainty, a risk/benefit assessment of a new drug (Pugsley *et al.* 2008).

Its future will depend, in part, upon the scientific and technological advances and regulatory challenges that envelop pharmaceutical development The scientific challenge facing Safety Pharmacology is to keep pace, to adapt, and to incorporate new technologies in the evaluation of new drugs in nonclinical models and identifying the effects that pose a risk to human volunteers and patients (Bass *et al*. 2004).

It was suggested to evaluate the respiratory system using different methodologies assessing RR and other measures of respiratory function (*e.g*. TV) or Hb saturation in O_2 (ICH 1997). To obtain reliable results, it is important to reduce to a minimum the potential effects of exterior factors that may affect the response of the animals. It was suggested by Savoie (1982), to use non-invasive methods since these methods are less stressful on the animals and do not require anaesthesia, analgesia or tranquilizer.

3.1. Non-invasive methods

3.1.1. Spirometry

Spirometry is the measurement of airflow into and out of the lungs (Wagner *et al.* 2006). It measures air movement and results are expressed in volume (Lumb 2006). It is the most common method used to evaluate lung function (Wagner *et al*. 2006). It is inexpensive, convenient, and easy to perform. It is used to determine among other things VC, the forced vital capacity, the forced expiratory volume (FEV), the peak expiratory flow rate, and the FEV at 25-75% of the volume expired.

The technique is widely used in humans for the diagnosis of lung disease, monitoring, evaluation of disability, or for public health surveys (Lockey *et al.* 2000). It is less suitable for animals as it requires subject cooperation (Coffman and Hessel, 2005). In spite of that difficulty, it has been used in animals for many years as a research tool. As early as 1923, spirometry was used in studies of energy metabolism in farm animals (Orr & Magee, 1923). It was also used in sheep (Edward, 1966), calf (Blaxter *et al.* 1953, Bureau *et al.* 2001), dogs (O'Toole *et al*. 2001) and cats and rabbits (Bartlett, 1957).

More recently it has been used in rodent to study for the development of rodent models of sulfur mustard-induced pulmonary injury (Weber *et al.* 2010). In anesthetized rodents, the possibility of using a two-sidearm tracheal cannula for measurements of respiratory airflow was investigated by Mortola and Noworaj (1983). This procedure appeared to be a suitable and very practical way of measuring mean airflow and tidal volume in anesthetized small animals, ranging in size from newborn rats to newborn puppies.

3.1.2. Body plethysmography

Recent emphasis on the benefits of noninvasive technology has renewed interest for the use of body plethysmography, among other things to analyze expiratory tidal flow patterns as a tool to assess airway obstruction. The main advantage of this approach is its non-invasiveness, allowing simple, rapid, and

repeatable measurements of several conscious animals at a time (Hantos & Brusasco 2002).

The whole-body plethysmography method is a non-invasive method to measure the respiratory function. Emka Technologies (Emka Technologies, USA, 307 Annandale Road, Falls Church, VA 22042, USA) has developed a system that can be used in freely moving laboratory animals such as rodents, guinea pigs, rabbits, dogs, pigs and monkeys. The manufacturer can also produce a modified system to allow evaluating respiratory parameters in conscious restrained or anesthetized rodents as well as in guinea pigs.

The plethysmograph has two chambers, each fitted with a pneumotachograph. The subject is placed in one of them (subject chamber) and the other remains empty (reference chamber).

A modification of the whole body plethysmography, the head out plethysmography, has also been used. Animals are gently placed in the body plethysmographs while the head of each animal protrudes through a neck collar into a ventilated head exposure chamber (Glaab 2007). The procedure has been used in sheep (Bedenice *et al.* 2004). Aerosols can be delivered directly through the head exposure chamber. Tidal flow measurement is made with a calibrated pneumotachograph and a differential pressure transducer attached to the top port of each body chamber. The amplified and digitized flow signals are integrated with time to obtain TV. From these signals several standard respiratory parameters, including TV, RR, consequently MV, time of inspiration and expiration, and mid-tidal expiratory flow (EF_{50}) can be derived from software analysis. The non-invasive measurement of EF_{50} was first described as an appropriate instrument to measure airway responsiveness in conscious mice by Vijayaraghavan *et al.* (1994). Moreover, EF_{50} is based on physiological principles and has a physical meaning [mL/s] that is directly related to airway resistance, thus enabling quantitative interpretation of airway changes between animals (Hantos *et al.* 2002).

The barometric plethysmography has been also used to study respiratory parameters. The procedure has been described by Jacky (1978). In that procedure, the subject rests in an environmental chamber, and respiration is indicated by barometric pressure oscillations proportional to tidal volume. It has been used in mice (Hamelmann *et al.* 1997; Vijayaraghavan *et al.* 1994), in rats (Johanson &Pierce 1971, Hamelmann *et al.* 1997), in guinea pigs (Pennock *et al.* 1979), and in monkeys (Twenhafel *et al.* 2009).

Plethysmography has an advantage over other procedures such as the gas dilution methods which fail to record the volume of any gas that may be trapped in an isolated compartment inside the lung, and therefore give a false-low value of FRC. During plethysmography, values are unaffected by such trapped gases (Sabyasachi 2007).

3.1.3. Pneumotachometry

Pneumotachometry is a procedure most commonly used to monitor ventilation during acute illness in human (Cannon *et al.* 2000). The most commonly used devices for this purpose are the Fleish and the Silvermann pneumotachometers, which function by creating a very slight resistance to air flow. Pressure differential across this resistance is proportional to, and in phase with, the speed of air movement (Waterhouse 2005). The flow sensing device is positioned in the mouth and the flow signal is integrated over time to compute volumes. The Fleish pneumotachometer remains the reference instrument but does not always cover the entire range of flows to be measured. Major advantages of pneumotachometers are their compactness and their possible use with an open breathing circuit. They are easier to use and to decontaminate than spirometer and are gradually replacing those (Hans-Ulrich & Bankier 2003).

3.1.4. Oscillometry

Forced oscillometry is a very attractive method because it is non-invasive and appears to be more sensitive to changes in lung functions than are

measurements of dynamic compliance and total pulmonary resistance. Flow and pressure are measured at the nares in response to impulses applied to the respiratory system (McGorum *et al.* 2007). It determines breathing mechanics by superimposing small external pressure signals on the spontaneous breathing of the subject (DuBois *et al.* 1956). Forced oscillometry is indicated as a diagnostic method to obtain reliable differentiated tidal breathing analysis.

The ability to measure lung function in similar ways in human and monkey studies is a particular advantage (Coffman & Hessel 2005). In primates the non-invasive forced oscillation technique is the preferred technique for measuring changes in pulmonary function (Gundel *et al.* 1992). This technique superimposes forced oscillations onto normal spontaneous breathing either at the airway opening or at the body surface, and measures respectively input or transfer impedance, from which airway resistance and compliance are calculated (Madwed & Jackson, 1997). In humans, the forced oscillation technique is used with subjects unable to cooperate, such as very young children, and for specific research purposes (Oostveen *et al*. 2003). Potential applications of oscillometry include paediatric, adult, and geriatric populations, comprising diagnostic clinical testing, monitoring of therapeutic regimens, and epidemiological evaluations, independent of severity of lung disease (Smith & Goldman 2005).

The technique has also been used in various animal species. In horses (Erck *et al*. 2003, 2004; Klein *et al.* 2006), it was, for instance, more sensitive than the oesophageal balloon method which is the conventional reference technique, for pulmonary function testing (Erck *et al.* 2003). It seems to be a practical and non-invasive pulmonary function test that may be useful in assessing subclinical changes in horses (Erck et al. 2004; Reinhold *et al.* 1996).

In new born calves, it was successfully used to evaluate their respiratory adaptation to extra-uterine life during the first 24 hours (Uystepruyst *et al.* 2000). It was also used in lamb (Pillow *et al.* 1996), dogs (Clercx *et al.* 2003), rabbits, (Peslin *et al*. 1994), guinea pigs (Sobh *et al.* 1997) and rats (Thamrin *et al.* 2005).

3.2. Invasive methods

Direct measurement of pleural pressure requires placement of a catheter in the pleural cavity. Fortunately, oesophageal pressure also provides an accurate estimate of pleural pressure (McGorum *et al.* 2007). Measurement of oesophageal pressure is a practical approach to measuring changes in intrathoracic pressure, for the evaluation of respiratory system mechanics in the assessment of pulmonary physiology and pathophysiology. The pressures across the lungs and chest wall can be measured, and these measurements, together with volume and flow, allow the respiratory system resistance and compliance to be partitioned into their pulmonary and chest wall components. Several approaches can be used to make measurements, including air-filled balloon catheters, liquid-filled catheters, and small transducers placed in the oesophagus (Murphy *et al.* 1998). When the subject is relaxed or under local anaesthesia, the catheter is carefully inserted through the nostril. When the subject feels that the tip of the device is touching the pharynx, a mouthful of water should be swallowed. The test consists of occluding a subject's airways at end-expiration while measuring oesophageal pressure and airway opening pressure during three to five spontaneous inspiratory efforts. (Qutayba *et al.* 2005).

Another approach for invasive assessment of airway function in rodents is the low-frequency forced oscillation technique. The procedure was derived from similar techniques used in humans and larger animals. It gives estimates of lung impedance, which can be considered the most detailed measurement of pulmonary mechanics currently available (Peslin & Fredberg 1986).

4. POSITIVE CONTROL PHARMACEUTICAL SUBSTANCES

Pharmaceutical substances with known effects were used as positive controls. Those substances included: albuterol, metacholine and remifentanil. It appears important in the literature review to present a summary of their respective pharmacology.

4.1. Albuterol

Albuterol is a selective ß$_2$-adrenergic agonist with pharmacological properties and therapeutic indications. It is administered either by inhalation or orally for the symptomatic relief of bronchospasm. When administered by inhalation, it produces significant bronchodilation within 15 minutes and effects are demonstrable for 3 to 4 hours (Hardman *et al.* 1996). Inhalation of a ß-adrenergic agonist is clearly the preferred therapy for bronchoconstriction *per se* (Fanta *et al.* 1986). Beta-adrenergic agonists are the only agents shown to be immediately effective in the setting of acute, severe asthma.

4.2. Methacholine

Methacholine chloride (acetyl-â-methylcholine) is a parasympathomimetic synthetic analog of acetylcholine. It stimulates muscarinic postganglionic parasympathetic receptors, causing bronchial smooth muscle constriction (Birnbaum & Barreiro 2007). Used by inhalation in humans for the diagnosis of bronchial hyperreactivity in asthma (Tulic *et al.* 1999) or chronic obstructive pulmonary disease, it is also recognized to induce reflex bronchoconstriction when administered into the bronchial artery (Wagner & Jacoby 1999).

Methacholine has a more prolonged action than acetylcholine because it is hydrolyzed by acetylcholine esterase at a considerably slower rate and is almost totally resistant to hydrolysis by nonspecific cholinesterase. Its selectivity is manifested by slight nicotinic and a predominance of muscarinic actions, the latter being most marked on the cardiovascular system (Hardman *et al.* 1996).

4.3. Remifentanil

Remifentanil is a potent opioid selective for μ-opioid receptors that produces intense analgesia very rapidly. It shares with other opioids respiratory depression, bradycardia, skeletal muscle rigidity, and reversibility by naloxone. In contrast to other short-acting opioids, remifentanil contains an ester linkage and so is metabolized by circulating and tissue nonspecific esterases (Hardman

et al. 1996). As a result, recovery time from remifentanil is rapid and almost independent of dose or duration of infusion (Egan *et al.* 1993).

5. PUBLICATIONS

FIRST ARTICLE:

RESPIRATORY SAFETY PHARMACOLOGY: CONCURRENT VALIDATION OF VOLUME, RATE, TIME, FLOW AND RATIO VARIABLES IN CONSCIOUS MALE SPRAGUE-DAWLEY RATS.

This manuscript has been submitted for publication to the Journal of Regulatory Toxicology and Pharmacology. The aim of this study was to evaluate the performance qualifications of the FlexiWare® system in conscious Sprague Dawley male rats following the administration of pharmacological substances with known effects on the respiratory system.

RESPIRATORY SAFETY PHARMACOLOGY: CONCURRENT VALIDATION OF VOLUME, RATE, TIME, FLOW AND RATIO VARIABLES IN CONSCIOUS MALE SPRAGUE-DAWLEY RATS.

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Abstract: 172 words

Text: 3985 words

References: 685 words

Abstract:

This study compares basic respiratory variables (rate, tidal and minute volumes) with time-, flow- and ratio-derived parameters obtained using head-out plethysmography in rats following administration of reference drugs (isotonic saline, 2.0 mL/kg, IV; albuterol, 400 µg/kg, inhalation; methacholine, 136 µg/kg, IV; and remifentanil, 14 µg/kg, IV) to identify respiratory variables with superior sensitivity. Paired t-tests by block-period, and analysis of covariance (ANCOVA) with baseline as covariate and *a posteriori* pair-wise comparisons using Dunnett's test were used. Variations in respiratory parameters observed over time justify the use of a control group in any respiratory safety pharmacology study for inter-groups comparison. Handling-, and slumbering-, induced perturbations were minimal. The system was sensitive and specific to detect changes in respiratory variables related to pharmacologically-induced bronchodilation, bronchoconstriction and central respiratory depression. The standard variables (respiratory rate, tidal and minute volumes) confirmed to be the cornerstone of respiratory safety pharmacology to detect pharmacological changes. Flow-derived parameters appeared as highly valuable complement for interpretation of respiratory response, whereas time- and ratio-derived parameters presented limited added value during interpretation.

Key words: ICH S7A, Rat, Conscious, Respiratory function, Monitoring qualifications, Validation.

1. Introduction

The respiratory system is assessed during safety pharmacology studies based on regulatory requirements for drug safety testing (Anon., 2001). The rat model is the preferred species in most drug development programs (Lindgren et al., 2008) with regards to its high genetic homogeneity, lower test material requirements, and abundant historical data. Various non-invasive and invasive methods are used to evaluate the effects of test substances on the respiratory function (Murphy et al., 1998; 2001; Sommer et al., 1998; Walker et al., 1997). Among these methods, plethysmography (Hoymann, 2007), pneumotachometry (Davies and Dunster, 2002), oscillometry (Hoffman, 2002), and esophageal catheterization (Eng et al., 1990) are used based on the various experimental requirements.

Minimizing the potential effects of external factors that may affect animal response is critical to obtain reliable respiratory data. Savoy et al. (1982) suggested the use of non-invasive methods which are less stressful to animals and do not require anesthesia, analgesia, or sedation. Head-out and whole body plethysmographs are accepted non-invasive respiratory monitoring methodologies and are widely used in safety pharmacology. The head-out plethysmograph is simple to use, and allows nearly natural breathing pattern (Hoymann, 2006). Recently, we have reported the effects of reference drugs on basic respiratory variables [tidal volume (TV), respiratory rate (RR), and minute ventilation (MV)] monitored with head-out plethysmograph in rats (Authier et al., 2009).

Validation of test systems is a process involving three qualifications tiers, namely installation (IQ), operational (OQ) and performance (PQ) qualifications. The accuracy of a measurement system is the degree of closeness of measurements of a quantity to its actual (true) value. Accuracy is usually provided by the supplier and is verified during IQ/OQ, which were outside of the scope of the present work. Regulatory considerations of the validation process (FDA, CFR 21 Part 11: electronic records and electronic signatures) were also outside of the focus of the current discussions. By conducting the PQ, our scientific interests were to test precision, *i.e.* repeatability and reproducibility, as well as sensitivity and specificity of the test system in the conditions that prevailed during the study. Therefore, the aims of the PQ evaluation were, respectively, 1) to determine the within-time stability of respiratory measurements before and after saline-placebo (negative drug, *i.e.* without any expected respiratory effect) administration with stable head-out plethysmography environmental conditions; to administer reference drugs (saline and positive drugs with known effects on the respiratory system) to conscious male Sprague-Dawley rats in order to test 2) inter-individuals reproducibility; and 3) concurrent validity. Criterion (concurrent) validity is a measure of how well one variable or set of variables predicts an outcome based on information from other variables. Concurrent validation was assessed by comparing basic respiratory variables (RR, TV, and MV) with time-, flow- and ratio-derived parameters to identify variables with superior sensitivity or affected specificity.

2. Materials and methods

2.1. Statement on use and care of animals and regulatory compliance

During the study, care and use of animals were conducted in accordance with principles outlined in the current Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (CCAC) and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). LAB Research, Inc.'s facility is CCAC and Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee before study initiation. Data were obtained under studies conducted in accordance with the Good Laboratory Practice (GLP) regulations of the United States Food and Drug Administration (21 CFR Part 58 and subsequent amendments).

2.2. Animal housing

The experimental population comprised thirty-two (32) specific pathogen-free male Sprague-Dawley rats (242–315 g, 59–65 days). Animals were obtained from Charles River (St-Constant, QC, Canada) and were group-housed (2-3 animals per cage) in polycarbonate cages equipped with filter top and water bottles. They were fed ad libitum with standard certified commercial rodent chow (Teklad Certified 18% Lab Rodent Diet #2018C) and provided with

reverse osmosis water. The animal room environment was controlled (targeted ranges: temperature 18-24°C, humidity 30–70%, 12 h light/12 h dark, 10–15 air changes/h). Temperature and relative humidity were monitored continuously.

2.3. Respiratory monitoring system

Respiratory variables were continuously recorded at a sampling frequency of 200 Hz. Respiratory function was monitored using head-out plethysmographs (Model PC-H2, SCIentific Respiratory Equipment Quebec (SCIREQ) Inc., Montreal, QC, Canada) with opaque headrest connected to a pneumotachometer (Model 8420 with a 5 L/min capacity, Hans Rudolph, MO, USA). The headrests were such that the nose of the rat was open to ambient air, but visual and auditory stimulations were attenuated, providing a more comfortable environment for the animals as supported by the regular breathing patterns observed and decreased resistance to the restraint in the plethysmograph. Airflows from rat pneumotachometers were monitored using differential pressure transducers (Model UT-PDP-02, 0.2 kPa nominal, SCIREQ Inc., Montreal, QC, Canada) connected to the plethysmographs and to the data acquisition matrix (In expose-08, Data Acquisition Controller 8 channels, SCIREQ, Inc., Montreal, QC, Canada) and a real-time respiratory analyzer (flexi-Ware 5.3.1, SCIREQ, Inc., Montreal, QC, Canada) software installed on a computer (Model PU-2.5 OptiPlex 745 Workstation, Dell, Dallas, TX, USA).

2.4. Positive and negative control drugs

Isotonic saline (0.9% sodium chloride, Baxter, ON, Canada), 400 μg/kg

albuterol (Ratiopharm, Inc., QC, Canada), 136 μg/kg methacholine (Sigma-Aldrich, ON, Canada) and 14 μg/kg remifentanil (Ultiva®, Abbott Laboratories Ltd., ON, Canada) were administered to conscious rats, at doses selected based on historical data, to induce moderate to severe respiratory effects (Authier et al., 2008; 2009).

Dose volumes for all intravenous agents were 2.0 mL/kg (all intravenous agents), and administered by tail vein injection using a butterfly catheter. Albuterol was administered by inhalation *via* a pressurized metered-dose inhaler with a holding chamber and mask. All administrations were performed with minimal handling to minimize interferences..

2.5. Experimental design

Animals were randomized based on body weight using assigned random numbers (Microsoft Office Excel 2003). A total of 8 animals in each group were dosed on 4 consecutive days. All animals (n=8) receiving the same treatment were dosed the same day. The treatment order was assigned to systematically change the sequences.

Body weight was recorded prior to randomization and on the dosing day. All animals were acclimated to the head-out plethysmograph restraint unit with opaque headrest. The animals were acclimated in the head-out plethysmograph for 4 days with increasing durations up to 2 1/2 h.

Within 30 min after animals were placed in the plethysmograph, pulmonary monitoring was initiated to establish pre-dosing baseline. After at least one hour

of baseline monitoring, animals were temporarily removed from the plethysmograph for dosing. Following dosing, animals were returned to the plethysmograph and pulmonary monitoring was continued for a period of approximately four hours. Data were collected continuously for the following eleven variables: RR, TV, MV, inspiratory time (IT), expiratory time (ET), time to peak expiratory flow, peak inspiratory flow, peak expiratory flow, mid-tidal expiratory flow (EF50), inspiratory:expiratory (I:E) ratio, and inspiratory to total breath (I:TB) ratio. At the end of the recording period, animals were returned to their cages.

2.6. Statistical Analysis

Data were continuously monitored at 200 Hz and were averaged in single 5 min block of data for the control and treated groups. The averaged data were grouped in four different blocks: block 1 (0-30 min pre-treatment), block 2 (31-60 min pre-treatment), block 3 (0-120 min post-treatment) and block 4 (121-240 min post-treatment). Data from each block were compared using repeated measures general linear model. *A priori* contrast analysis for each drug was conducted using a paired t-test based on values averaged during 60 min (12 measures at 5-min interval) prior to dosing, and up to 4 h (48 measures at 5 min interval) post-treatment. Finally, a repeated measures two-way analysis of covariance (ANCOVA) with baseline score as a covariate, comparing saline to positive drugs, was performed to detect intergroup difference, with *a posteriori* pair-wise comparisons using Dunnett's test (Vickers, 2001). A limitation of the study design was the use of the same control group with IV administration for the three

treated groups including albuterol, which was given by inhalation. We assumed that inhalation and IV administration would have comparable effects on the respiratory variables *per se*.

Alpha threshold was set at 0.05, mean (SD) values (except when difference is notified) are presented, and SAS version 9.1 for Windows (SAS Institute, Inc., Cary, NC, U.S.A.) was used (Littell et al., 2006). The analysis initially included standard respiratory parameters, RR, TV, and MV, followed by the three time-derived variables, the three flow-derived variables, and the two ratios. Characterization of stability (or consistency between repeated measures under the same conditions) was based on data provided from the placebo group (n=8). Therefore, within-time repeatability was assessed in the placebo group before (pre-dosing period) and after saline administration. Stability was also tested during the pre-dosing period of the drug treated groups. Inter-individuals reproducibility, or test system responsiveness, was tested by systematically evaluating the individual response to positive drug administration. Maximal percentages of change were calculated from the average value for each variable measured during the pre-treatment period. Concurrent validity focused on the response obtained with RR, TV and MV compared with other variables. Sensitivity and specificity were assessed by comparing the response obtained with these three variables and the parameters of interest using *all-or-none* binary estimation.

3. Results

3.1. Saline

None of the respiratory parameters monitored showed statistically significant difference within the pre-treatment period, or between the pre-treatment period and the first two hours post-treatment with saline (block 3). However, RR (p<0.0001) and MV (p=0.003) significantly decreased during the last two hours post-treatment (block 4) when compared to the pre-treatment period. A compensatory increase in TV (p=0.03) was observed over the same period. Parallel to the decrease in RR, all three within breath time variables, IT (p<0.0001), ET (p=0.004), and time to peak expiratory flow (p<0.0001), significantly increased. If peak inspiratory flow significantly (p=0.002) decreased during block 4, this was not the case for peak expiratory flow and EF50. Both I:E and I:TB ratios slightly but significantly decreased during block 4 (p=0.02).

When post-treatment measures were compared to the pre-treatment averages, an increase in TV (Fig. 1) and MV was observed at the first time-point post-dosing (T5 min). The decreases in RR (-19%) and MV (-18%) noted during block 4 were mainly related to the values collected around T210 min post-dosing. Alteration of the three times variables (+55% in IT at T220; +19% in ET at T210; +48% in time to peak expiratory flow at T220), peak inspiratory flow (-21% at T210), and on both ratios (+41% in I:E and +8% in I:TB at T220) were

noted at the same time point. Seven out of 8 rats presented the same pattern, suggesting that this was a reproducible phenomenon.

3.2. Albuterol

Over time, the albuterol-treated group showed similar changes to the control group for all respiratory variables at block 4, in comparison to the pre-treatment period. However, significant differences were observed at block 3 for TV (p=0.0018), MV (p=0.009), peak inspiratory flow (p<0.0001), and peak expiratory flow (p=0.005) (Table 1).

The ANCOVA revealed an increase in TV at T5 (p=0.0106), and a decrease at T15 (p=0.001), and T20 min (p=0.011) post-dosing (Fig. 1). MV increased at T5 (p=0.01) and RR decreased at T25 (p=0.032) and T30 min (p=0.0274) post-dosing (Fig. 2). Time to peak exploratory flow and IT did not significantly change, but ET was increased at T25 (p=0.0354) and T30 (p=0.024). Peak inspiratory flow was increased in the albuterol group compared to the saline group at T5 (+25%) and T10 (p=0.028). In contrast, peak expiratory flow and EF50 were not significantly altered. I:TB ratio significantly decreased at T5 (p=0.0034), T20 (p=0.0422), T60 (p=0.0071), and T90 (p=0.0287), whilst the I:E ratio only reached statistical significance at T60 (p=0.02). Seven out of 8 rats presented the same pattern, suggesting that this was a reproducible phenomenon. The described changes were marked in 2 rats.

3.3. Methacholine

Over time, the methacholine-treated group presented alterations in all respiratory variables, except I:E and I:TB, at blocks 3 and 4 compared to the pre-treatment (Table 1).

The ANCOVA revealed a significant decrease in RR (Fig. 2 and 3) and MV at T10, T20, T90, T150, and T180 min post-dosing associated to a compensatory increase in TV at T5 and T10 (Fig. 1). Time to peak expiratory flow (+108%, $p<0.02$), IT (+107%, $p<0.05$), and ET (+39%, $p<0.03$) were increased at T5 and T10, as well as T60, T90, T150, and T180 for ET. Peak inspiratory (+18%, $p=0.01$) and expiratory (+26%, $p<0.0001$) flows were initially increased at T5. Subsequently, peak inspiratory flow decreased at T60 ($p=0.028$) and T90 (-17%, $p=0.011$) while peak expiratory flow (Fig. 4) and EF50 also decreased at T30, T60, T90 (-21% for both), T150 and T180 ($p<0.05$). I:E ratio was not significantly altered, whereas I:TB was reduced at T60 ($p=0.0294$) and T90 ($p=0.01$). In general, the respiratory response to methacholine in rats appeared more heterogeneous. A comparable respiratory pattern was observed in 5 out of 8 rats, and particularly the intensity of the response induced by methacholine was high in 3, moderate in 2, and variable in the remaining rats.

3.4. Remifentanil

The remifentanil-treated group showed significant alterations in all respiratory parameters, except I:E and I:TB, at blocks 3 ($p=0.7835$, and $p=0.9784$, respectively) and 4 ($p=0.1752$, and $p=0.0773$) compared to the pre-treatment, as

well as peak inspiratory flow (p=0.25) and EF50 (p=0.0673) at block 3 (Table 1).

The ANCOVA revealed a decrease (-13%, p=0.04) in RR (Fig. 2) associated to a compensatory increase (+11%, p=0.03) in TV at T5 (Fig. 1). Time to peak expiratory flow (+44%, p=0.0002), IT (+35%, p=0.004), and ET (+61%, p<0.0001) were increased at T5. Peak expiratory flow was not modified, while EF50 was significantly (-11%, p=0.02) reduced at T5 and peak inspiratory flow decreased at T10 (p=0.0165) and T15 (-36%, p=0.0008). The I:E ratio was not significantly altered, but I:TB was reduced at T10 (p=0.0003), T20 (p=0.0361), and T25 (p=0.013). Six out of 8 rats presented the same respiratory pattern, suggesting that this was a reproducible phenomenon.

4. Discussion

As previously reported with this monitoring modality (Hoymann, 2007; Murphy et al., 1998), animals appeared calm and relaxed, as indicated by constant and normal respiratory parameters over the first 4-hour period of follow-up. Results obtained in the control group (saline) suggest that head-out plethysmographs resulted in minimal stress to the animals, whereas no respiratory effects were observed during the 60-min baseline and the first 2-hour (block 3) post-treatment. Transient handling-induced perturbations were initially noted after placement in the head-out plethysmograph. Our results differ from the 60 to 90 min perturbations observed after intraperitoneal methylcellulose vehicle

administration observed by Delaunois *et al*. (2009). Result differences may be attributed to variation of monitoring environment or handling.

However, during block 4, animals demonstrated a decrease in RR associated to a compensatory increase in TV, but insufficient to avoid a decrease in MV (MV = RR * TV). The increases in IT, ET, and time to peak expiratory flow reflect the prolongation of inspiratory and expiratory phases and the corresponding RR decrease. Flow measurement was less sensitive to detect such change in ventilation, as only peak inspiratory flow presented a significant decrease in this period. Finally, both ratios were minimally affected by the increased depth in tidal ventilation and associated reduction in RR. Such effects could be related to a possible slumbering of the rats, occurring after a period exceeding 4 hours (most significant results noted at T210 min post-dosing) in the plethysmograph. Our results reveal modest but significant variations of respiratory parameters over time in saline treated animals, which justify systematic inclusion of a control group for inter-group comparison in respiratory safety pharmacology studies. Multiple statistical analysis strategies are acceptable in the presence of an occasion effect and the ANCOVA with baseline as covariate is considered as an appropriate statistical plan to detect the potential respiratory changes induced by test articles (Vickers, 2001). The respiratory variables typically included in the safety pharmacology core battery (RR, TV, and MV) confirmed their sensitivity to detect slight changes related to rat slumbering. Measurements of time components are complementary to the change in RR, and flow

measurements appeared less sensitive to detect respiratory changes. Finally, I:E and I:TB ratios were minimally altered, which suggested limited sensitivity.

Trend graphs on Fig. 1 and 2 illustrate the interrelation between TV and RR changes after administration of positive drugs. Albuterol, a $ß_2$-agonist, promotes airway relaxation causing predominantly effects on bronchial smooth muscle (Ameredes and Calhoun, 2009). In humans, albuterol is administered by inhalation and produces a bronchodilation (Pesola and Coelho D'Costa, 2004), leading to an increase in TV and MV (Sorbini et al., 1984). Similar responses were obtained when tested in dogs and monkeys (Authier et al., 2009). The effect has a rapid onset, which is typical for this route of administration with a short duration (Dhand et al., 1999). As observed in other species, albuterol resulted in a significant increase in TV. The repercussions on MV were only present at one time-point (T5), and peak inspiratory flow was increased at T5 and T10. Consequently, I:TB was reduced, as we had hypothesized that the increase in flow rate could lead to a diminution of the inspiratory phase, hence the effect on I:TB, even if IT was not sensitive enough to detect this change. These results suggest that albuterol-mediated bronchodilation, and subsequent decrease in airways resistance are mainly detected by changes in TV and peak inspiratory flow.

Methacholine chloride (acetyl-β-methylcholine) stimulates muscarinic postganglionic parasympathetic receptors, causing bronchial smooth muscle constriction (Birnbaum and Barreiro, 2007). Intravenous methacholine acts

mainly on the airway producing an increase in airway resistance whereas inhaled methacholine alters the mechanical properties of both the airways and the lung tissues (Petàk et al., 1997). In rats, the bronchial response to a high dose of methacholine was characterized with a transient increase in TV associated with a persisting decrease in RR and MV. Concurrently, IT, ET, and time to peak expiratory flow were all increased, with longer effects for ET. Of all three tested positive drugs in this study, methacholine IV injection induced the most important respiratory effects. Fig. 3 and 4 illustrate individual variability of RR and peak expiratory flow in response to methacholine. Methacholine-induced bronchoconstriction is mild in humans (Fujimori et al., 1996) and monkeys (Authier et al., 2009) leading to decreased TV and MV. The respiratory response to methacholine-induced bronchoconstriction was different in rats (increased TV, decreased RR and MV). Despite opposite changes in TV and MV, the decrease in peak inspiratory flow, and most importantly in peak expiratory flow and EF50 support an increase in airways resistance. Recently, the validity of EF50 as a non-invasive measurement of bronchoconstriction in rats was questioned (Ewart and Valentin, 2009). Although, EF50 was reduced after bronchoconstriction with methacholine in our model, it was also decreased after remifentanil which induces a central respiratory depression without recognized effects on bronchoconstriction. Interestingly, EF50 was decreased after bronchoconstriction (statistically significant) and bronchodilation (not statistically significant). A decrease in EF50 should not be systematically

attributed to bronchoconstriction and direct measures of pulmonary resistance should be considered.

Remifentanil is an ultrashort-acting and potent μ-opioid leading to central respiratory depression (Ansermino et al., 2005). Remifentanil is unique among currently available opioids because its ester structure renders it susceptible to hydrolysis by non-specific blood and tissue esterases (Feldman et al., 1991). In consequence, the decreased firing from respiratory centers leads to hypoventilation, characterized by a drop in RR, potentially reaching apnea in case of overdose. In the current rat study, we observed similar transient and acute bradypnea associated with a compensatory increase in TV, which was even more evident with a data analysis at 1-min intervals (Authier et al., 2009). Concurrently, IT, ET, and time to expiratory flow increased considerably at T5. Peak inspiratory flow and EF50 were transiently reduced at the same time-points, further supporting the severity of the hypoventilation.

The three basic respiratory variables (RR, TV, MV) were altered by all treatments, either a bronchodilator (albuterol), a bronchoconstrictor (methacholine) or a central respiratory depressor (remifentanil). The time-derived variables were almost systematically and inversely related to the changes observed on RR. The flow derived parameters detected changes in ventilation, indicative of a certain level of specificity, but these parameters also demonstrate the limitations of the model which does not include a direct measure of aurway resistance. Globally it would be very difficult to distinguish different levels of

bronchodilatation , or even a bronchoconstriction from a bronchodilation. It remains that core battery respiratory safety pharmacology is aimed to identify possible respiratory liabilities that could later be investigated with follow-up studies.

These results confirm the suitability of head-out plethysmography in rats for respiratory safety pharmacology as previously reported by Hoymann (2006). In addition, data obtained following negative control (saline) suggested a limit of 3 to 4 hours before slumbering alters respiratory patterns in conscious rats. Variations over time in the control group justify the systematic use of a control group in any respiratory safety pharmacology study for inter-groups comparison. Moreover, the system was sensitive and specific to detect changes in respiratory parameters related to pharmacologically-induced bronchodilation (albuterol), bronchoconstriction (methacholine) and central respiratory depression (remifentanil). In the case of ultrashort-acting agents, such as remifentanil, the analysis sensitivity could be improved by shortening the epoch of data analysis (analysis on 1 min averages instead of 5 min averages). The three parameters (RR, TV, and MV) outlined in the ICH S7A guideline confirmed to be the cornerstone of respiratory safety pharmacology data interpretation providing the most sensitive detection of changes. The addition of within-breath time parameters was not essential to detect drug effects but offered complementary tools to interpret changes in RR. Evaluated ratios did not prove to be particularly useful in the interpretation of pharmacological respiratory effects in the current

study. Nevertheless, evaluation of peak inspiratory / expiratory flow and EF50 appears as a valuable complement for interpretation of respiratory response, as previously suggested (Hoymann, 2007). Peak inspiratory flow was altered by the three positive control drug challenges, and peak expiratory flow and EF50 were particularly altered by the increase in airways resistance.

5. Acknowledgements

The authors thank the personnel of LAB Research, Inc. for providing the infrastructure and technical resources required during study conduct. Grateful acknowledgement is made to the Canada Foundation for Innovation, On-going new opportunities grant # 9843 attributed to ET for infrastructure support.

Conflict of interest statement: The authors declare that there are no conflicts of interest.

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Table 1
Effect of positive and negative control substances on some respiratory parameters [mean (SD)] in conscious male Sprague–Dawley rats.

Test Substances	Respiratory rate (b/min)	Tidal volume (mL/b)	Minute volume (mL/min)	Inspiratory time (sec)	Mid-tidal expiratory flow (mL/sec)
Saline					
Pre-Rx	200.16 (27.43)	1.39 (0.11)	264.46 (28.85)	0.20 (0.03)	-12.33 (1.27)
Post-Rx 0-2 h	198.49 (38.66)	1.40 (0.11)	261.18 (48.93)	0.21 (0.06)	-12.46 (1.64)
Post-Rx 2-4 h	182.54 (38.44)*	1.44 (0.14)*	249.86 (53.91)*	0.24 (0.06)*	-12.45 (1.27)
Albuterol					
Pre-Rx	171.81 (37.05)	1.62 (0.11)	273.11 (60.08)	0.22 (0.06)	-11.33 (2.57)
Post-Rx 0-2 h	162.15 (34.39)†	1.65 (0.17)*	255.15 (57.02)*,†	0.23 (0.03)	-11.04 (2.46)
Post-Rx 2-4 h	157.94 (31.45)*	1.66 (0.11)*,†	250.22 (39,60)*	0.24 (0.03)*	-11.38 (2.69)
Methacholine					
Pre-Rx	208.06 (39.57)	1.31 (0.08)	263.04 (46.58)	0.19 (0.03)	-11.99 (1.78)
Post-Rx 0-2 h	166.97 (31.59)*,†	1.47 (0.25)*,†	232.85 (41.83)*†	0.24 (0.11)*,†	-10.72 (1.75)*,†
Post-Rx 2-4 h	161.73 (44.29)*,†	1.48 (0.11)*	228.73 (52.72)*,†	0.25 (0.06)*	-10.59 (2.77)*,†
Remifentanil					
Pre-Rx	188.70 (31.31)	1.52 (0.17)	271.62 (56.51)	0.20 (0.03)	-11.94 (1.95)
Post-Rx 0-2 h	170.27 (39.88)*,†	1.61 (0.14)*,†	260.90 (51.79)*	0.23 (0.06)*,†	-11.50 (2.72)†
Post-Rx 2-4 h	171.37 (35.38)*	1.59 (0.20)*	257.04 (45.59)*	0.23 (0.03)*	-11.69 (2.63)

*Intragroup significant difference (within-time evaluation by post-dosing blocks comparison to pre-treatment period)

†Intergroup significant difference (ANCOVA comparison to the placebo-controlled group *via* Dunnett's test at each significant time-point)

Figure legends

Photo 1. A-The SCIREQ computerized system; **B-**A rat in place in the head-out plethysmograph.

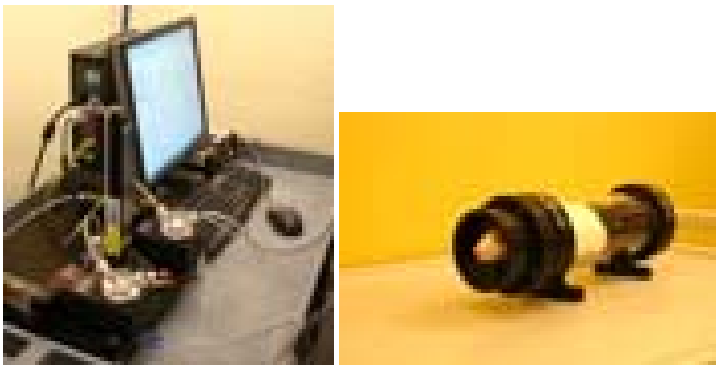


Fig. 1. Tidal volume (TV) evolution in each treated group from baseline to 60-minute post-dosing. Histogram is presenting group mean ± SEM at each selected time-point, whereas the trend curve is a mobile average (period of 2).

Saline (2.0 mL/kg IV); Albuterol (400 µg/kg inhalation); Methacholine (136 µg/kg, 2.0 mL/kg IV); Remifentanil (14 µg/kg, 2.0 mL/kg IV). ANCOVA with baseline score as a covariate, comparing saline to positive drugs, was performed to detect intergroup difference, with *a posteriori* pair-wise comparisons using Dunnett's test (* = $p < 0.05$).

Trend curve clearly highlights the significant within-time change in TV after saline injection at T5. Compared to the saline control group, albuterol inhalation increased significantly TV at T5, and decreased it at T15 and T20. Methacholine induced a significant increase at T5 and T10. Remifentanil increased significantly at T5.

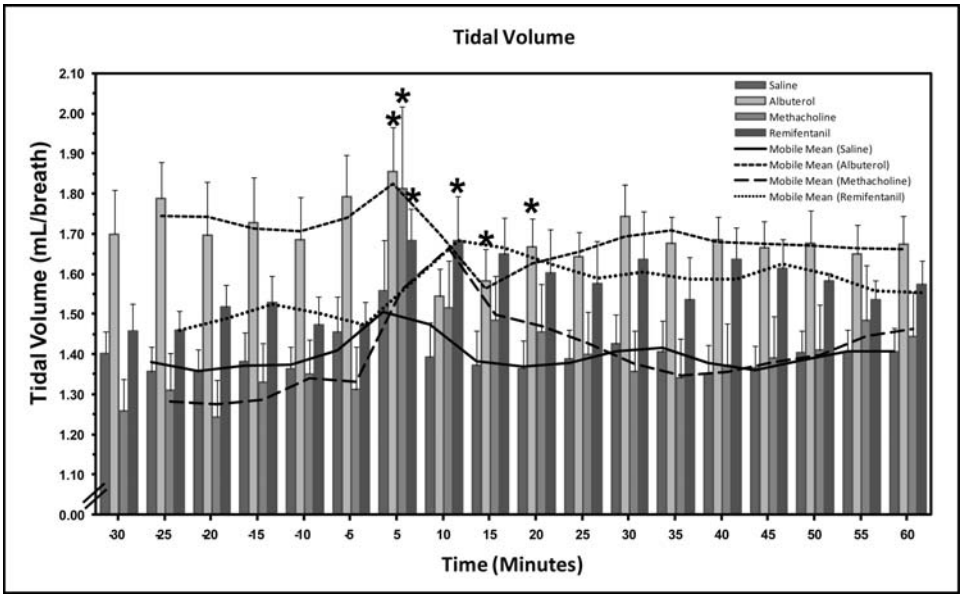


Fig. 2. Respiratory rate (RR) evolution in each treated group from baseline to 60-minute post-dosing. Histogram is presenting group mean ± SEM at each selected time-point, whereas the trend curve is a mobile average (period of 2).

Saline (2.0 mL/kg IV); Albuterol (400 µg/kg inhalation); Methacholine (136 µg/kg, 2.0 mL/kg IV); Remifentanil (14 µg/kg, 2.0 mL/kg IV). ANCOVA with baseline score as a covariate, comparing saline to positive drugs, was performed to detect intergroup difference, with *a posteriori* pair-wise comparisons using Dunnett's test (* = $p < 0.05$).

Trend curve shows the within-time stability of RR in the saline control group. Albuterol induced an initial increase in RR (which was not statistically significant) followed by a significant decrease at T25 and T30. Methacholine induced a significant (at T10 and T20) and persisting decrease in RR. Remifentanil induced a transient decrease in RR, significant only at T5.

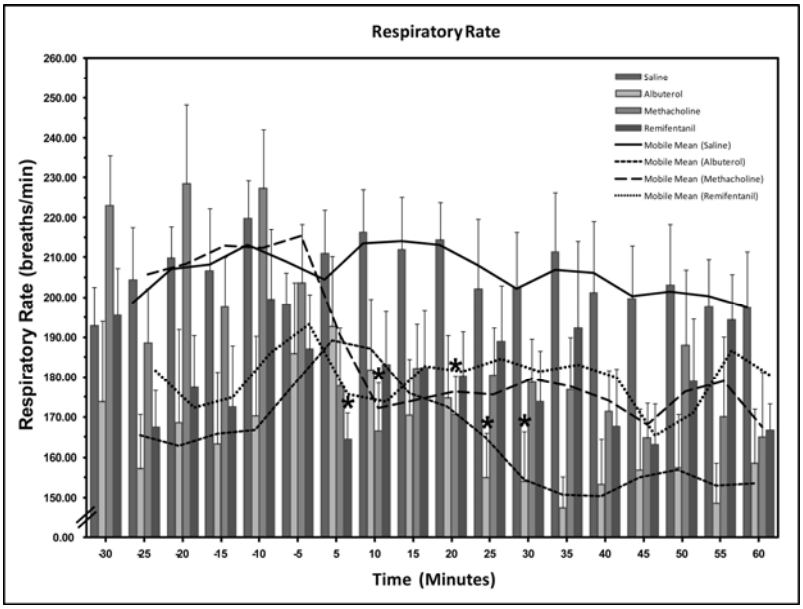


Fig. 3. Individual changes (n=8) observed in respiratory rate (RR) of rats following administration of methacholine (136 μg/kg, 2.0 mL/kg IV).

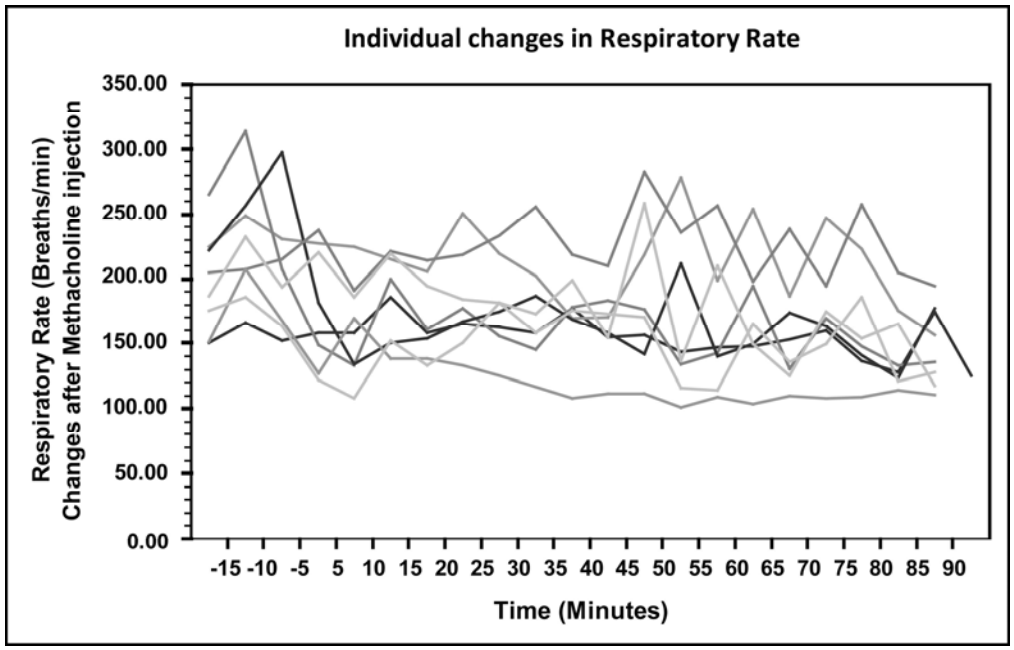
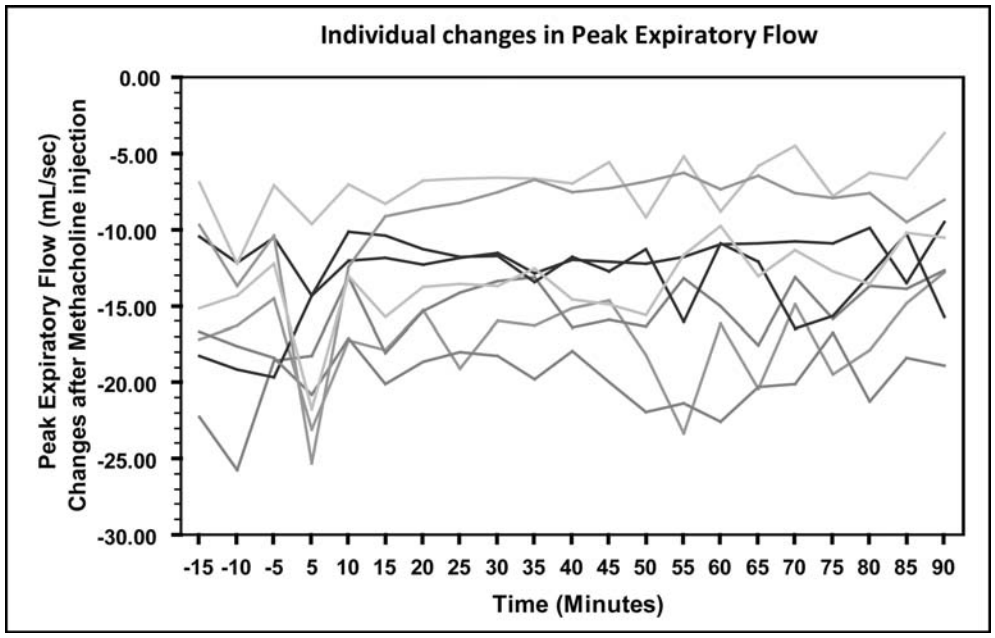


Fig. 4. Individual changes (n=8) observed in peak expiratory flow (PEF) of rats following administration of methacholine (136 μg/kg, 2.0 mL/kg IV). The values are indicated as negative, but the changes were described on the absolute values (*i.e.* what was described as a decrease in PEF was for example the change from -15 to -10 mL/sec).



SECOND ARTICLE:

RESPIRATORY SAFETY PHARMACOLOGY: POSITIVE CONTROL DRUG RESPONSES IN SPRAGUE-DAWLEY RATS, BEAGLE DOGS AND CYNOMOLGUS MONKEYS.

This manuscript has been published in the Journal of Regulatory Toxicology and Pharmacology. The aim of this study was to compare the respiratory changes induced by drugs with pharmacologically-known effects, on 40 conscious Sprague Dawley male rats, 18 Beagle dogs (6 males and 12 females), as well as 8 Cynomolgus monkeys (4 males and 4 females).

Respiratory Safety Pharmacology: Positive Control Drug Responses in Sprague-Dawley Rats, Beagle Dogs and Cynomolgus Monkeys

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Suggested section: **Safety Evaluation**

Short title: RESPIRATORY SAFETY IN THREE SPECIES

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ABSTRACT

Rats are most frequently used to fulfill ICH S7A requirements for respiratory safety pharmacology. We hypothesized that the models used to assess respiratory safety pharmacology present different ventilatory responses to bronchoconstriction, bronchodilation and respiratory depression. Respiratory monitoring was performed with head-out plethysmographs for rats, masks for dogs and bias airflow helmets for monkeys. Respiratory rate (RR), tidal volume (TV) and minute volume (MV) were recorded. Forty rats, 18 dogs and 8 monkeys were acclimated to the respiratory monitoring equipment. Animals received saline (IV), albuterol (inhalation), methacholine (IV) and remifentanil (IV). Albuterol increased TV in all species. Methacholine decreased TV and MV in monkeys. In dogs, methacholine increased TV, RR and MV. In rats, methacholine increased TV and decreased RR. Remifentanil induced central respiratory depression in all species with decreased MV, except in rats. Dogs presented a biphasic response to remifentanil with hypoventilation followed by delayed hyperventilation. The monkeys presented similar responses to humans which may be due to biologic similarities. Dogs and rats presented clinically significant ventilatory alterations following positive control drugs. Although, the response to bronchoconstriction in dogs and rats was different from humans, the two species presented ventilatory changes that highlight the potential adverse effect of test articles.

Keywords: Respiratory safety, ICH S7A, rat, monkey, dog, conscious, tidal volume, respiratory rate, minute volume

1. INTRODUCTION

The ICH S7A Guideline defines the safety pharmacology core battery including cardiovascular (CVS), central nervous system (CNS) and respiratory (U.S. Food and Drug Administration, 2001). The guideline states that "*respiratory rate and other measures of respiratory function (e.g. tidal volume or hemoglobin oxygen saturation) should be evaluated*". A recent industry survey revealed that respiratory rate, tidal volume and minute volume are included in 98 to 100% of core battery and supplemental respiratory safety pharmacology studies (Lindgren et al. 2008). Study design in safety pharmacology is constantly evolving prodded by emerging technologies (Hoymann, 2007; Murphy *et al.*, 1998; Murphy *et al.*, 2001) and integrated drug development where *in vitro*, preclinical and clinical safety testings share a common goal, drug approval. Sensitivity of the model should be commensurate with the risk in our drug development industry where resources are scarce and time is felt by drug companies with aging drug patents. Blood gases, reported to have limited sensitivity (due to complex compensatory mechanisms) to detect drug-induced adverse effects (Authier *et al.,* 2008), and RR are occasionally used as sole *in-vivo* markers of preclinical respiratory safety. With the addition of histopathological assessments, these two *in-vivo* biomarkers (blood gases and RR) are, in some cases, considered acceptable for respiratory safety testing (*e.g.* some oncology indications).

When non-clinical and clinical reasons for drug development discontinuation are combined, respiratory adverse effects are less frequent than

CVS or CNS (Valentin & Hammond, 2008). Moreover, respiratory safety pharmacology is less frequently frontloaded (performed earlier in the drug development process) than CVS and CNS which may suggest a perceived lower risk of respiratory liability in comparison to the other two systems included in the safety pharmacology core battery.

The rat is used as the preferred species for respiratory safety in most investigational new drug (IND) programs (Lindgren et al. 2008). Advantages to the use of rats are numerous including high genetic homogeneity which reduces variability, lower test material requirements, abundant historical data and ethical considerations which favour the use of a phylogenically lower laboratory animal species. On the flip side, the use of rats may present disadvantages including limited genetic diversity which may not be representative of the human patient population. Respiratory anatomy, physiology and pathology in rats are relatively different from humans, an adaptive response to their respective normal habitat. Large laboratory animals such as dogs and monkeys present respiratory system characteristics which could be considered closer to humans.

IND submissions often require that a small and a large animal species be selected for toxicology studies. CVS safety pharmacology assessments are most frequently performed in large animals (Lindgren *et al.*, 2008) using dogs (Gauvin *et al.*, 2006) or monkeys (Authier *et al.*, 2007). Respiratory assessments to fulfil S7A can be performed using minimally invasive methodologies in toxicology studies or in large animals used for CVS safety pharmacology without the need to use additional animals. As large animals present biologic similarities with

human patients, inclusion of respiratory measures in large animal studies may increase the clinical relevance of these investigations. The current project presents the ventilatory responses of common respiratory safety pharmacology models to bronchodilation, bronchoconstriction and respiratory depression using well characterized pharmaceutical agents.

2. MATERIALS AND METHODS

2.1. Statement on use and care of animals and regulatory compliance. During the study, care and use of animals were conducted in accordance with principles outlined in the current Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). LAB Research Inc.'s facility is AAALAC accredited. Data were obtained under studies conducted in accordance with the Good Laboratory Practice (GLP) regulations of the United States Food and Drug Administration (21 CFR Part 58 and subsequent amendments).

2.2. Animal housing and preparation. The experimental population comprised forty (40) male Sprague-Dawley rats (242 to 315g, 59 to 65 days), eighteen (18) Beagle dogs (6 males and 12 females, 8 to 18 kg, 1 to 4 yrs) and eight (8) cynomolgus (*Macaca fascicularis*) monkeys (4 males and 4 females, 2.5 to 4.6 kg, 3 to 5 yrs old). The animal room environment was controlled and monitored continuously (targeted ranges: temperature 21 ± 3°C, humidity 30-70%, 12 hours

light, 12 hours dark, 10-15 air changes per hour). Rats were fed a standard rodent chow *ad libitum* (Teklad Certified 18% Rodent Diet #2018C). Dogs received a standard certified commercial dog chow (400 g of Harlan Teklad Certified 25% Lab Dog Diet #8727C) over a 24-hour feeding period. A standard certified commercial primate chow (Teklad Certified Global 25% Primate Diet # 2055C) was made available to each monkey twice daily. Clinical signs were evaluated at cage side at least once daily, and a detailed clinical examination was performed at transfer and once weekly throughout the studies.

2.3. Respiratory Monitoring System. Respiratory rate (RR), tidal volume (TV) and minute volume (MV) were recorded continuously at a sampling frequency of 200 Hz in all species. Respiratory function was monitored in rats using head-out plethysmographs (Model PC-H2, SCIREQ Inc., Montreal, QC, Canada) with opaque headrest connected to a pneumotachometer (Model 8420 with a 5 L/min capacity, HANS RUDOLPH, MO, U.S.A.). The headrests were such that the nose of the rats was open to ambient air but visual and auditory stimulations were attenuated providing a more comfortable environment for the animals. Airflow signals from rat pneumotachometers were monitored using precision differential pressure transducers (Model UT-PDP-02, 0.2 kPa nominal, SCIREQ Inc., Montreal, QC, Canada) connected to the data acquisition matrix (In expose-08, Data Acquisition Controller 8 channels, SCIREQ Inc., Montreal, QC, Canada) and a real-time respiratory analyzer (flexiWare 5.3.1, SCIREQ Inc., Montreal, QC, Canada).

Respiratory monitoring was performed in conscious Beagle dogs as previously described (Authier *et al.*, 2008). In brief, canine respiratory function was monitored using a computerized system composed of a data acquisition controller (DAC 8, Scientific Respiratory Equipment Quebec Inc. (SCIREQ), Montreal, QC, Canada) using a real-time respiratory analyzer (flexiWare 5.1, SCIREQ Inc., Montreal, QC, Canada) connected to pneumotachometer (Model 3719 with a 100L/min capacity, HANS RUDOLPH, MO, U.S.A.) with individual heater controllers (HANS RUDOLPH, MO, U.S.A.) connected to a face mask.

Respiratory function was monitored in conscious cynomolgus monkeys using a computerized system composed of a data acquisition controller (In expose-08, Scientific Respiratory Equipment Quebec Inc. (SCIREQ), Montreal, QC, Canada) connected to a computer (OptiPlex GX280 Workstation, DELL, Dallas, TX, U.S.A.) with a real-time respiratory analyzer (flexiWare 5.1, SCIREQ Inc., Montreal, QC, Canada). The respiratory hardware included pneumotachometer (Model 3500 with a 35L/min capacity, HANS RUDOLPH, MO, U.S.A.) with individual heater controller (HANS RUDOLPH, MO, U.S.A.). Monkeys were acclimated to a restraining chair and a transparent plexiglass helmet (LOMIR BIOMEDICAL, Notre-Dame-de-l'Île-Perrot, QC, Canada) with a biais airflow (inExpose pump module 2.5 lpm, SCIREQ Inc., Montreal, QC, Canada) and continuous helmet pressure monitoring (Model UT-PDP-25, 2.5 kPa nominal, SCIREQ Inc., Montreal, QC, Canada). Helmet pressure was maintained neutral to ambient pressure.

Rats, dogs and monkeys were acclimated to respiratory monitoring on three (3) different occasions before initiation of treatment. Rats and dogs that did not tolerate the respiratory monitoring were replaced by animals kept in the same experimental conditions. Respiratory monitoring was well tolerated by all monkeys assigned to the study and no replacement was required.

2.4. Positive and negative control drugs. Saline (BAXTER, ON, Canada), albuterol (RATIOPHARM Inc, QC, Canada), methacholine (SIGMA-ALDRICH, ON, Canada) and remifentanil (Ultiva®, ABBOTT LABORATORIES Ltd., ON, Canada) were administered to conscious rats, dogs and monkeys at doses selected based on historical data obtained in previous studies conducted at LAB Research and adjusted for body surface area (BSA) to induce slight and moderate to severe respiratory effects as presented in Table 1. The dose volume was 2.0 mL/kg for rats, and 0.2 mL/kg for dogs and monkeys (all intravenous agents). Albuterol was administered using a pressurized metered-dose inhaler with a holding chamber and a mask. Monitoring was initiated at least 15 min before dosing in all species. Respiratory data during the first 20 min after administration was used for comparison between species given the rapid onset of pharmacological effects with selected agents.

2.5. Data analysis. Data were averaged every 5 min and results following positive control drugs compared with saline administration. For rats, one (1) min averages at peak effect were also calculated. Normality of distribution was evaluated using the Shapiro-Wilk test. The Levene test was used to examine the homogeneity of group variances. When both of these tests were found to be non-

significant, analysis of variance (ANOVA) was considered appropriate. Whenever the overall group differences were shown significant (F-Test for ANOVA), then pair-wise comparisons were conducted using Dunnett's test for ANOVA. Comparisons among dose levels at each timepoint were done using a T-test and including the Satthertwaite method in presence of heterogenous group variances. Results are presented as mean values ± standard error of the mean (SEM).

3. RESULTS

Average RR after saline treatment was 175 ± 15 b/min, 16.4 ± 3.3 b/min and 50.5± 3.7 b/min in rats, dogs and monkeys, respectively. Average TV after saline treatment was 1.30 ± 0.07 mL, 217 ± 75 mL and 42 ± 17 mL in rats, dogs and monkeys, respectively. Mean MV following saline administration were 0.257 ± 0.004 L/min, 4.050 ± 0.407 L/min and 2.054 ± 0.315 L/min in rats, dogs and monkeys, respectively.

Albuterol administered by inhalation induced an increase in TV (Fig. 1) in rats, dogs and monkeys when using an analysis with 5 min averages for a 20 min monitoring period compared with saline treatment. Changes to RR were not statistically significant (Fig. 2) in any species during the 20 min monitoring period following treatment, while MV was significantly increased in dogs and monkeys (Fig. 3).

Monkeys presented a decrease in TV ($p<0.01$) and MV ($p<0.01$) following administration of methacholine with a trend to a compensatory

increase in RR at higher doses (Fig. 4). In contrast, respiratory monitoring in dogs differed with the dose of methacholine: at low dose (2 μg/kg), only MV presented a statistically significant change associated with a decrease in TV and an increase in RR at T10 min; at high dose (8 μg/kg) dogs presented an initial increase in RR ($p<0.01$) followed by an increase in TV ($p<0.05$) associated to a decrease in RR and a sustained increase in MV ($p<0.05$) (Fig. 5). In rats, systemic administration of methacholine resulted in dose-dependent effects: at low dose (28 μg/kg), no statistically significant effect was observed (Fig. 6), but at high dose (136 μg/kg), methacholine induced a significant increase in TV ($p<0.01$) with a decrease in RR ($p<0.05$) (Fig. 6).

As expected for a potent mu agonist opioid, remifentanil induced a significant respiratory depression in all three species (Table 2). Rats presented an initial and transient (at T5) decrease in RR, immediately compensated by a transient (at T10 and T15) increase in TV, without significant change in MV. When using one (1) min averages starting at pharmacological onset, RR (-37.4% ± 4.2%) and MV (-32.6% ± 5.3%) were significantly decreased ($p<0.01$) in rats following 14 μg/kg remifentanil administration. Dogs presented a biphasic response with hypoventilation followed by a delayed phase of hyperventilation with increased RR. Panting was noted in some dogs when adverse respiratory effects were seen which complicated respiratory analysis with this species. In monkeys, apnea was observed in 3 out of 7 monkeys at high dose (6.4 μg/kg), which prompted reversal of the opioid effects with naloxone (0.4 mg/mL, IV).

4. DISCUSSION

Species differences are recognized by regulators and dictate selection of maximum safe starting dose during initial clinical trials in healthy volunteers (U.S. Food and Drug Administration, 2005). Allometric correlations have been reported between species for metabolic rates but also for physiological parameters such as heart rate (West *et al.*, 2002), cardiac output, respiratory rate and ventilation (Lindstedt & Schaeffer, 2002). Allometric correlation between body weight and MV has been recognized for several decades (Guyton, 1947; Stahl, 1967). Inter-species correlations are recognized *de facto* in toxicology which uses models from multiples species to predict the human response. Allometric scaling of respiratory parameters is also a central exercise in inhalation toxicology studies for dose estimation. A formula ($V(m)=0.608\ BW^{0.852}$) to estimate respiratory MV adapted to laboratory animals (mouse, rat, dog and monkey) was recently published by the Association of Inhalation Toxicologists (AIT) (Alexander *et al.*, 2008). When using the recent AIT formula, the predicted MV (rats 0.191 ± 0.003 L/min; dogs 5.227 ± 0.225 L/min; monkeys 1.759 ± 0.100 L/min) were closer to actual respiratory measurements following saline administration than the predicted MV calculated (rats 0.166 ± 0.002 L/min; dogs 3.846 ± 0.196 L/min; monkeys 1.368 ± 0.074 L/min) with the Bide formula ($V(m)=0.499\ BW^{0.809}$) which is commonly used for dose calculation in inhalation toxicology studies (Bide *et al.,* 2000). These results support the superior predictive value of the recent AIT formula to estimate MV

when compared with the Bide formula. This closer correlation between predicted and measured MV using the AIT formula supports the use of the later in our experimental conditions. The importance to evaluate standard formulas with in-house data was demonstrated for QT correction formulas (Tattersall *et al.*, 2006). Similarly, allometric formulas used in dose calculation for inhalation toxicology studies benefit from qualification using actual MV values recorded in experimental conditions that prevail in each laboratory.

In humans, systemic administration of albuterol induces a significant increase in TV and MV (Sorbini *et al.*, 1984) which is thought to result from an increased metabolic rate and serum lactate (Tobin *et al.*, 2006). Similar to humans, monkeys and dogs presented a significant increase in TV and MV, while only increase in TV reached statistical significance in rats when compared with Saline. The duration of effects was longer in monkeys when compared to dogs at equivalent doses (0.1 mg/kg in dogs and 0.2 mg/kg in monkeys). This could be related to a greater respiratory reserve in dogs which resulted in a rapid compensation. Albuterol by inhalation induces tachycardia with a decrease in systolic pressure in beagle dogs (Petruska *et al.*, 1997). Similarly, rats presented tachycardia following inhaled albuterol at doses of 84 µg/kg (4.8 times lower than the dose used in the current study). Doses up to 7.5 times higher than the high dose (0.2 mg/kg) used in this study did not produce significant cardiovascular effects in cynomolgus monkeys. Cardiovascular effects of albuterol, also reported in healthy volunteers (Corea *et al.*, 1984), may contribute to respiratory changes when using this positive control drug in respiratory safety

models and in humans. Differences in ventilatory responses observed between the three models may be due to species specific sensitivity to cardiovascular and/or respiratory effects. Cardiopulmonary dependency is an emerging concept in safety pharmacology that often requires monitoring of the respiratory and cardiovascular systems simultaneously in the same animals to identify correlation between the two systems.

Methacholine at the high dose (8 μg/kg in dogs and 13.5 μg/kg in monkeys) induced diametrically opposite effects with a significant increase of MV in dogs while monkeys presented a decreased MV. Whereas TV was consistently decreased in monkeys at high dose methacholine, in dogs, TV was initially slightly reduced as previously reported (Savoy *et al.*, 1982) and associated with an abrupt increase in RR, followed by a second phase of respiratory response including increase in TV and return toward baseline values for RR. Such results suggest that the dog is highly responsive to the bronchoconstrictive effects of methacholine resulting in an abrupt increase in RR. Healthy humans are reported to present a decreased MV and TV (Fujimori K *et al.*, 1996) similar to the response observed in monkeys. Similar to dogs, the rats presented a response different from humans and monkeys with an increase in TV and a decrease in RR at the doses used in the current study. It remains that all three (3) species presented significant ventilatory changes following bronchoconstriction with methacholine and are considered suitable models to detect the presence of respiratory liability. Core battery safety pharmacology studies should identify potential adverse effects in human patients. The response

to bronchoconstriction, although different between species, will yield the same conclusion; identification of a potential respiratory (adverse) effect in humans which will trigger appropriate monitoring during early clinical trials.

Remifentanil, a potent mu-agonist opioid, leads to respiratory depression in humans (Smith *et al.*, 1997), which translates into decreased MV. Expected effects were observed in all species, but in rats the depression was more evident with the analysis at one (1) minute interval. Indeed for rats, analysis at onset of pharmacological effects with one (1) min averages revealed higher sensitivity to detect changes after remifentanil administration. The difference in statistical results between one (1) and five (5) min averages highlights the importance of post-acquisition data analysis which needs to be tailored to each test article in order to capture pharmacological effects. On the other hand, adapting statistical analysis *at posteriori* to capture an unknown pharmacodynamic response is a challenging issue in regulatory toxicology where any modification to the analysis plan would raise concerns on potential bias.

Results from the current study suggest that the ventilatory response to bronchoconstriction in the monkey is closer to humans. These pharmacodynamic similarities correlate with anatomic, physiologic and histologic characteristics of monkeys that resemble humans while dogs and rats present lower level of homology with humans. Monkeys (Dungworth *et al.*, 1975), dogs (Takenaka *et al.*, 1998) and humans (Saetta *et al.*, 1994) have several generations of respiratory bronchioles while rats have either no respiratory bronchioles of a single generation (Tyler & Julian, 1991; Saetta *et al.*, 1994). The number of

alveolar pores, which facilitates collateral ventilation, is similar in dog, monkey and human alveolus but lower in rats (Port *et al.*, 1977). Submucosal glands are observed throughout the bronchial tree of dog (Takenaka *et al.*, 1996), monkeys (El-Bermani & Grant, 1975) and humans (Scott, 1973) while rats do not have bronchial glands (Jeffery, 1983). Respiratory neural organization in monkeys, dogs and rats is comparable to humans (Kastner & Gauthier, 2008) with respiratory centers (inspiratory, expiratory, pneumotaxic and apneustic) located in the medulla oblongata and multiple nervous effectors controlling ventilation such as the phrenic and intercostal nerves (King, 2005). Despite central similarities the pulmonary innervation presents differences between species. The rat lung innervation presents significant differences when compared with monkeys (El-Bermani, 1978) while lung innervation in dogs is comparable to monkeys (Knight *et al.*, 1981). Will anatomical and physiological similarities between monkeys and humans translate into increased predictive value with this species? If so, will increased predictive value alter the decision making process during drug development? The answer resides in an integrated risk assessment of the toxicology testing plan. Despite differences between humans and rats, the rat model is widely accepted in pharmaceutical research (Tschernig *et al.*, 2008) and this species remains predictive of the patient response. Clinically significant alterations to respiratory parameters were noted in rats with all positive control drugs used in this study supporting the use of the three species for respiratory safety investigations.

The current study holds some limitations and complete dose-response curves would be needed to compare species sensitivity. It remains that dose levels that were used reliably induce bronchoconstriction, bronchodilation and respiratory depression given the well-characterized positive control drugs that were selected. As observed in the current study, dogs occasionally present panting when stressed or in response to drug induced adverse effects. Panting during respiratory monitoring acclimation in dogs triggers exclusion of individuals. When present, panting decreases accuracy of the ventilatory measures and increases artefacts due to excessively rapid ventilation of the respiratory dead space. Occasional panting is an inconvenient of the canine model and the rat can be preferred for respiratory safety testing when these two species are selected for toxicology studies. In contrast, the cynomolgus monkey maintains a tidal breathing pattern and the inclusion of non-invasive respiratory (ventilatory) investigations in toxicology studies may present some advantages over ventilatory assessments in rats.

Acknowledgements

Simon Authier is a *Ph.D.* student funded by the Natural Sciences and Engineering Research Council of Canada Graduate Scholarships and the Faculty of Graduate and Postgraduate Studies of Université de Montréal, Québec, Canada.

Eric Troncy is a member of a New Emerging Team program (#108291) of the Canadian Institutes of Health Research / Heart and Stroke Foundation of Canada and Discovery Grant (#327158-2008) of the Natural Sciences and Engineering Research Council of Canada.

The authors would like to thank Marilene Paquet, D.V.M., M.V.Sc., Dipl. A.C.V.P., Veterinary Comparative Pathology Services Comparative Medicine & Animal Resources Centre at McGill University for her support in species comparison of the respiratory system.

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Table 1

Dose levels of positive control drugs

	Route	Rats	Dogs	Monkeys
Saline	IV	--	--	--
Albuterol (µg/kg)	Inhalation	400	100	100
				200
Methacholine (µg/kg)	IV	28	2	3.4
		136	8	13.5
				68.0
Remifentanil (µg/kg)	IV	14	4	3.4
				6.8

Table 2 – Respiratory parameters following remifentanil (5 min averages starting at injection)

		Dose	Respiratory Rate (b/min)		Tidal Volume (mL)		Minute Volume (mL/min)	
Control Articles		µg/kg	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
Sprague-Dawley Rats	(n=8)	14	188.7 ± 10.4	164.4* ± 7.3	1.517 ± 0.069	1.683* ± 0.089	271.6 ± 13.5	262.4 ± 12.0
Beagle Dogs	(n=7)	4	15.8 ± 2.3	11.6* ± 0.8	270.7 ± 19.4	243.2 ± 24.3	3913 ± 440	2339** ± 193
Cynomolgus Monkeys	(n=8)	3.4	49.2 ± 3.2	39.6 ± 2.5	39.4 ± 3.5	23.0** ± 3.2	1864 ± 163	911** ± 165
	(n=7)[a]	6.8	50.4 ± 4.4	41.7 ± 3.4	41.6 ± 3.9	28.4* ± 4.5	1945 ± 143	1297* ± 287

* $p<0.05$, ** $p<0.01$

[a] Effects of remifentanil was reversed with naloxone IV for 3 out 7 animals due to severe apnea.

Figures

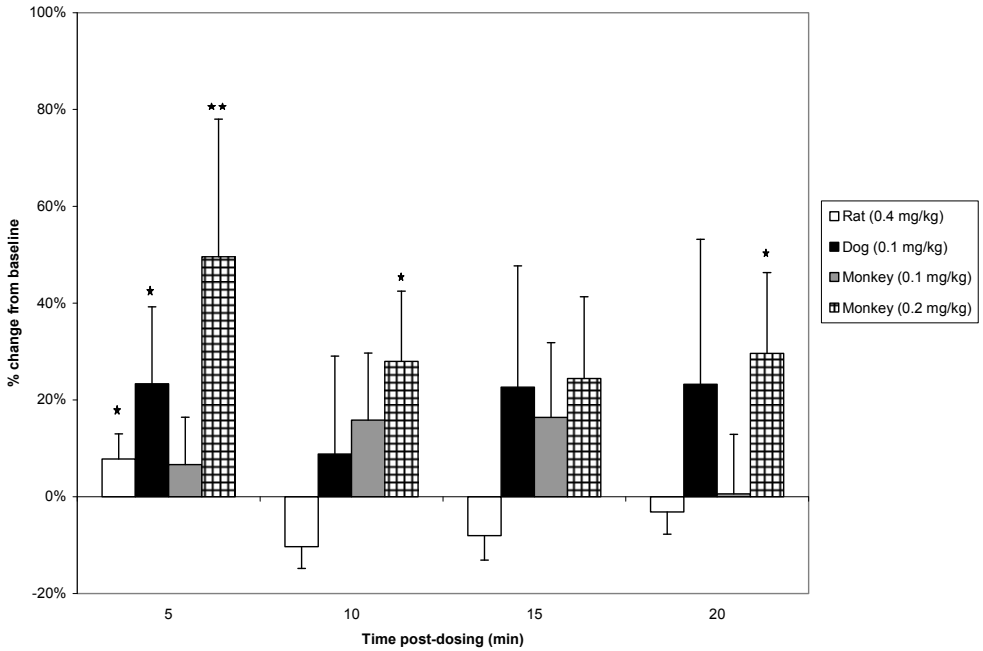


FIG. 1. Tidal volume (TV) after albuterol administered by inhalation to Sprague-Dawley rats (n=8), Beagle dogs (n=5) and cynomolgus monkeys (n=8). Overall group difference when compared with saline was significant for rats (p<0.05), dogs (p<0.05) and monkeys at 0.2 mg/kg (p<0.01).

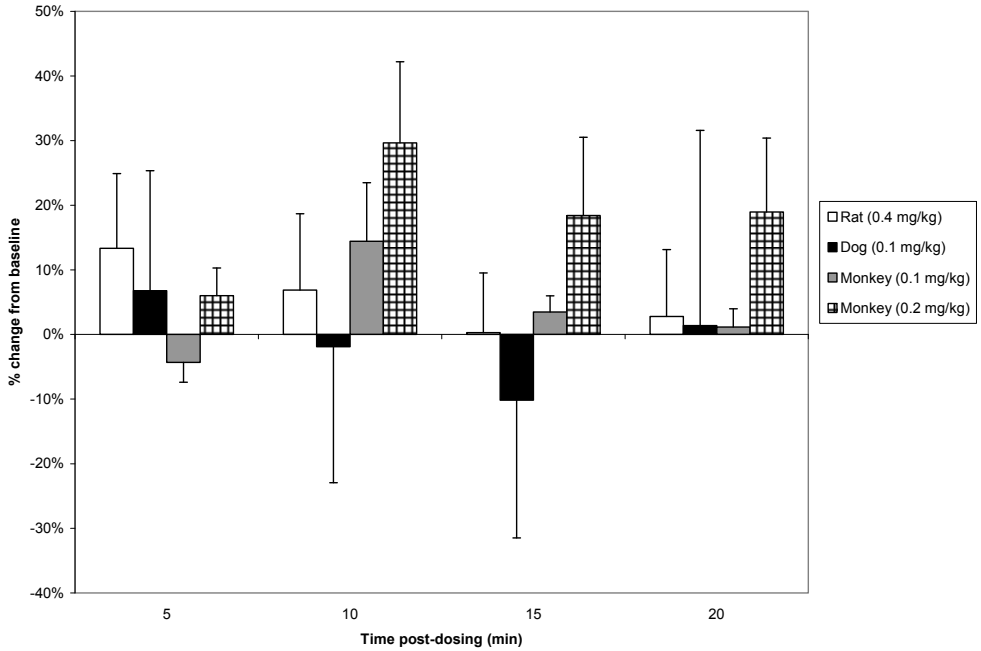


FIG. 2. Respiratory rate (RR) following albuterol administered by inhalation to Sprague-Dawley rats (n=8), beagle dogs (n=5) and cynomolgus monkeys (n=8). Overall group difference when compared with saline using ANOVA was not significant for any of the 3 species during the 20 min monitoring period with 5 min averages.

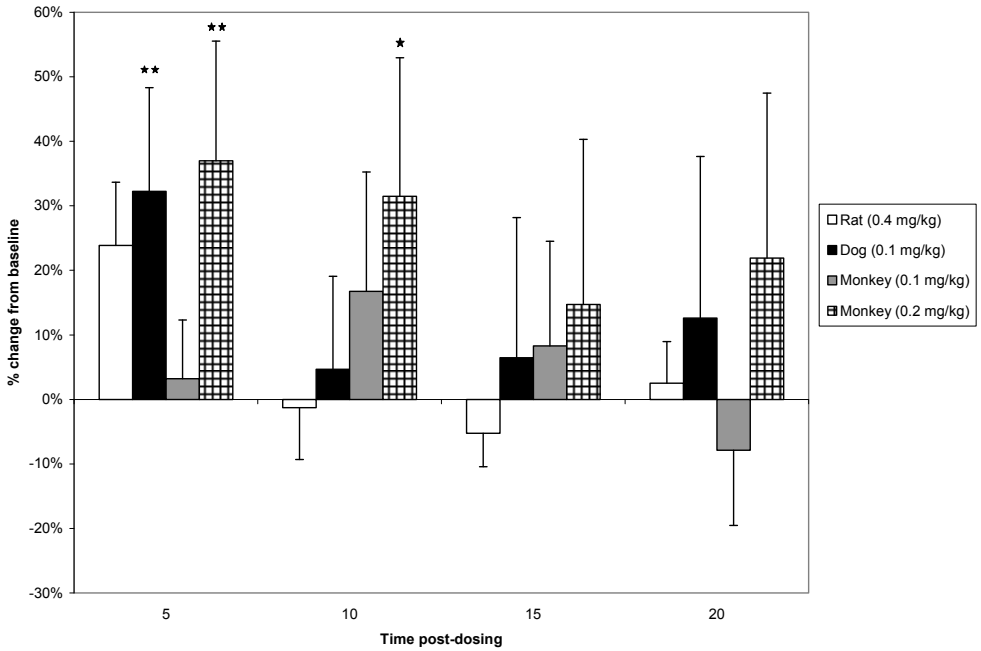


FIG. 3. Minute volume (MV) following albuterol administered by inhalation to Sprague-Dawley rats (n=8), beagle dogs (n=5) and cynomolgus monkeys (n=8). The overall group difference was statistically significant when compared with saline for dogs ($p<0.01$) and monkey at 0.2 mg/kg ($p<0.01$), while overall group difference did not reach statistical significance in rats when compared with saline.

** = $p < 0.01$.

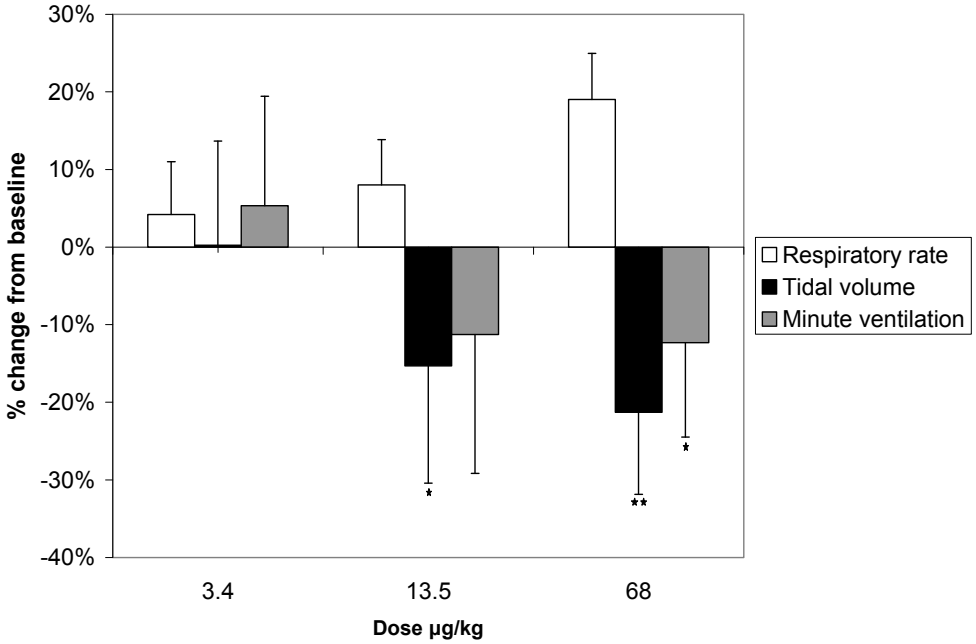
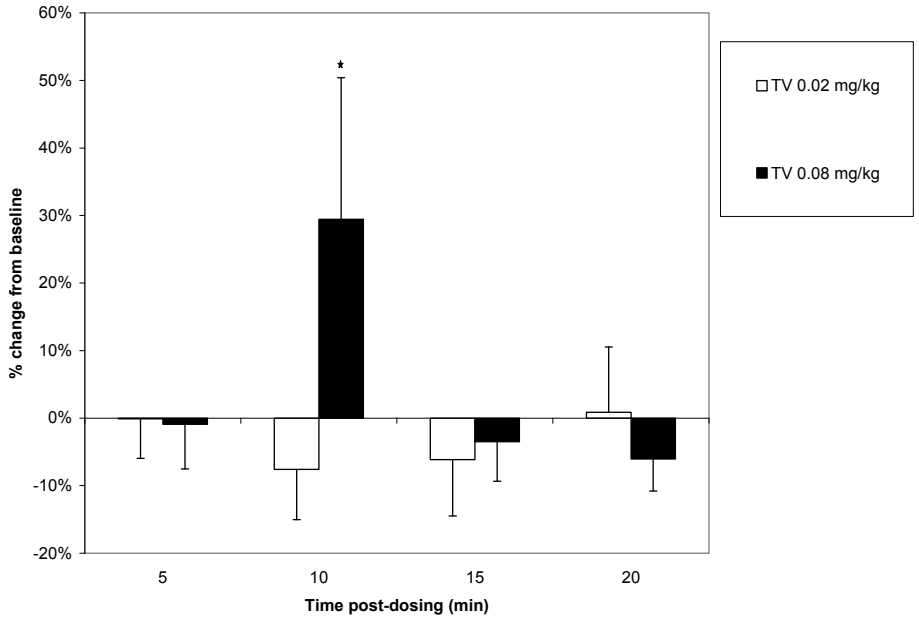


FIG. 4. Respiratory monitoring following methacholine bolus administration (IV) to cynomolgus monkeys (n=8). Overall difference was significant for tidal volume (p<0.01) and minute volume (p<0.01) and significance at each dose is presented above. A trend to compensatory increase in respiratory rate was observed following methacholine administration at 13.5 and 68 µg/kg.

* = $p < 0.05$, ** = $p < 0.01$.

A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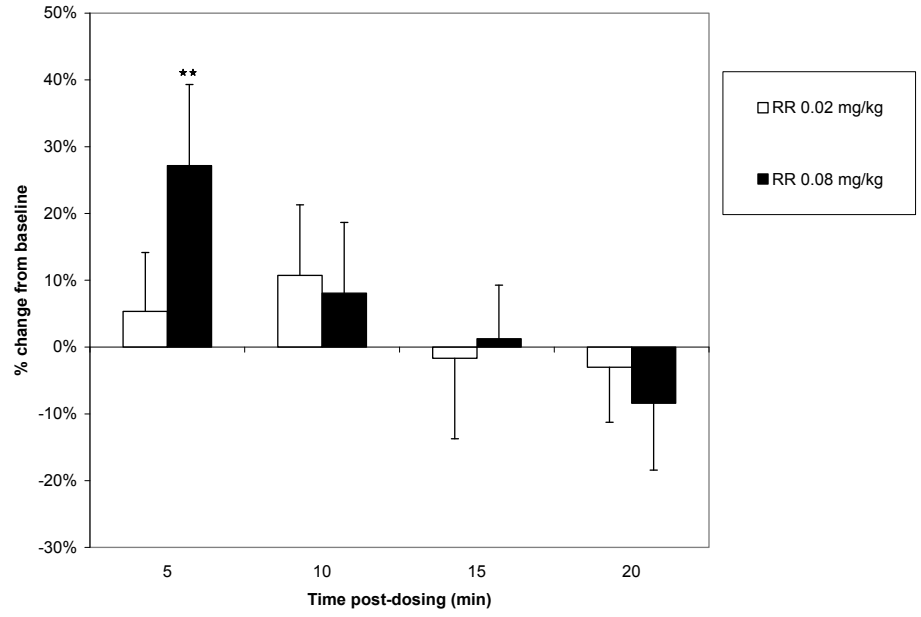
Time post-dosing (min)
TV 0.02 mg/kg
TV 0.08 mg/kg

B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RR 0.08 mg/kg

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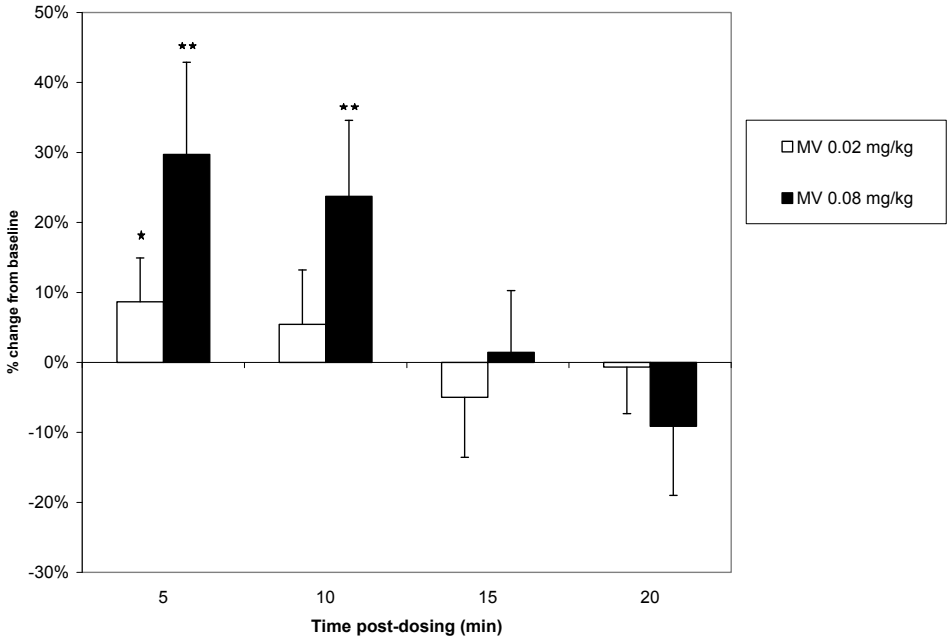

FIG. 5. Tidal volume (5A), respiratory rate (5B) and minute volume (5C) following methacholine bolus administration (IV) to beagle dogs (n=8). The overall difference was significant for MV at 2 µg/kg (p<0.01) and for all 3 parameters at 8 µg/kg (RR, p<0.01; TV, p<0.05; MV, p<0.05). At 8 µg/kg, a sustained increase in MV is explained by a biphasic response, with an initial increase in RR, followed by an increase in TV while RR is returned toward baseline.

* p<0.05; ** p<0.01

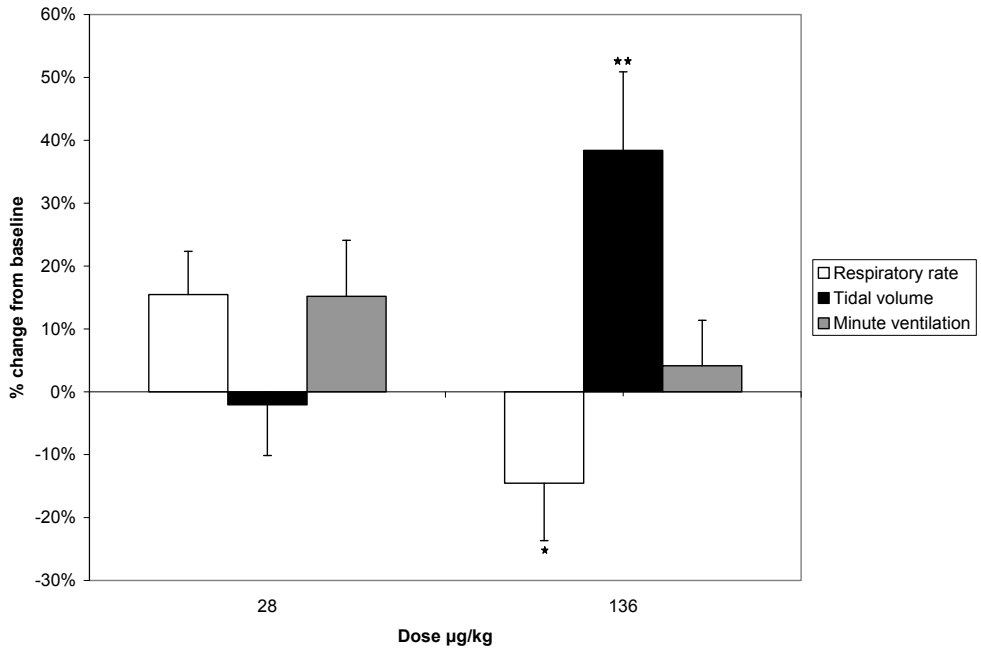

FIG. 6. Respiratory monitoring (5 min average at pharmacological onset) following methacholine bolus administration (IV) to Sprague-Dawley rats (n=8). When compared with saline, RR was decreased (p<0.05) and TV was increased (p<0.01) after methacholine at 136 µg/kg. The changes in RR (p=0.07) and MV (p=0.11) at 28 µg/kg did not reach statistical significance.

* = $p < 0.05$, ** = $p < 0.01$.

6. DISCUSSION AND CONCLUSION

The objective of the first study was to evaluate the performance qualifications of the FlexiWare® system in conscious Sprague-Dawley male rats following the administration of pharmacological substances with known effects on the respiratory system.

In studies using laboratory animals, to obtain reliable results, it is important to address the potential effects of exterior factors that may affect the response of the animals. Savoy *et al.* (1982) suggested the use of non-invasive methods which are less stressful to animals and do not require anaesthesia, analgesia, or sedation. In our study, the respiratory function was monitored using head-out plethysmographs. It is a non-invasive procedure requiring no anaesthesia, analgesia, nor sedation. It was well tolerated by the animals. Indeed the animals appeared calm and relaxed as indicated by constant and normal respiratory parameters over the first 4-hour period of follow-up. Results obtained in the control group (saline) suggest that head-out plethysmographs resulted in minimal stress to the animals, whereas no respiratory effects were observed during the 60-min baseline and the first 2- hour (block 3) post-treatment.

The FlexiWare® system version 5.3.1® used could detect the response of the rats following inoculation of the pharmacological substances used. We also demonstrated that this system could detect the response of the Beagle dogs and Cynomolgus monkeys following administration of the similar substances (Authier *et al.* 2009).

In the rat model used in this study, as well as in the Beagle dogs and in the Cynomologus monkeys, the system responded very well. The three basic parameters (RR, TV, and MV) outlined in the ICH S7A guideline confirmed to be the cornerstone of respiratory safety pharmacology data interpretation providing the most sensitive detection of changes. The system was sensitive and specific to detect changes in respiratory parameters related to pharmacologically-induced bronchodilation (albuterol), bronchoconstriction (methacholine) and

central respiratory depression (remifentanil). In the case of ultra-short-acting agents, such as remifentanil, the analysis sensitivity could be improved by shortening the epoch of data analysis (analysis on 1 min averages instead of 5 min averages), as particularly demonstrated for rats (Authier *et al*. 2009). The use of a more sensitive statistical method could also help to evaluate the effects of the ultra-short-acting agents. The addition of within-breath time parameters (IT, ET, and time to peak expiratory flow) was not primordial to detect drug effects but offered complementary tools to interpret changes in RR.

We have also evaluated more flow-derived parameters such as peak inspiratory flow, peak expiratory flow, and mid-tidal expiratory flow (EF50), as well as ratio-derived variables, *e.g.* inspiratory:expiratory (I:E) ratio and inspiratory to total breath (I:TB) ratio. Flow-derived variables appeared as highly valuable complement for interpretation for respiratory response. The ratio-derived parameters did not prove to be particularly useful in the interpretation of pharmacological respiratory effects in the current study. Nevertheless, evaluation of peak inspiratory / expiratory flows and EF50 appears as a valuable complement for interpretation of respiratory response, as previously suggested (Hoymann, 2007). Peak inspiratory flow was altered by the three positive control drug challenges, and peak expiratory flow and EF50 were particularly affected by the increase in airways resistance.

The three animals species used in this study (rats, Beagle dogs and Cynomologus monkeys) adapted well to the apparatus. However each species presented some particularities.

In the rat model, the duration of the testing period seem to be critical. Indeed, the animals showed a normal breathing pattern during the first three to four hours of testing. However, passed that period, unexpected breathing patterns were observed in both control and treated animals. Therefore, this makes interpretation of drug-induced effects more complex over this period. Furthermore, the data collection method raised a question that needs to be addressed. In the case of a drug producing a rapid action of a short duration (*e.g.*,

remifentanil), the effect of the drug cannot be detected when block data (average data collected during 2 hours post treatment) were compared with block data from control animals collected during the same period.

In the canine model we had observed that there is presence of occasionally panting when dogs were stressed or in response to drug-induced adverse effects. It is an important inconvenient when present. Panting decreases accuracy of the ventilatory measures and increases artefacts due to excessively rapid ventilation of the respiratory dead space.

In contrast, the cynomolgus monkey maintains a tidal breathing pattern and the inclusion of non invasive respiratory (ventilatory) investigations in toxicology studies may present some advantages.

Albuterol, considered the most widely used ß2-agonist for asthma, has an efficacy, safety and selectivity profile that makes it the drug of choice as rescue therapy in acute asthma exacerbations and symptoms. Albuterol promotes airway relaxation causing predominantly effects on bronchial smooth muscle (Ameredes & Calhoun 2009). In monkeys and dogs an increase in TV and MV were observed. However in rats, a significant increase in TV and peak inspiratory flow were noted leading to a diminution in inspiratory phase. The repercussions in MV were only present at one time point. These results suggest that albuterol-mediated bronchodilation, and subsequent decrease in airways resistance is mainly detected by changes in TV and peak inspiratory flow.

Methacholine chloride (acetyl-â-methylcholine) is a parasympathomimetic synthetic analog of acetylcholine. It stimulates muscarinic postganglionic parasympathetic receptors, causing bronchial smooth muscle constriction (Birnbaum & Barreiro 2007). The MV and TV were decreased in monkeys as similar effects in humans (Fujimori *et al.* 1996). In MV, an opposite effect was noted in dogs compared to monkeys. In dogs, TV was initially slightly reduced and associated with an abrupt increase in RR, followed by a second phase of respiratory response including an increase in TV, MV and return toward baseline values for RR. These suggest that the dog is highly responsive to the

bronchoconstrictive effects of methacholine. In rats the response was different from monkeys, with an increase in TV and a decrease in RR and MV. A decreased response in flow parameters (peak inspiratory flow and peak expiratory flow) support an increase in airway resistance. So, the analysis with complementary flow-derived variables appears as highly beneficial in rats. It remains, that all three (3) species presented significant ventilatory changes following bronchoconstriction.

In the case of remifentanil, an ultra short acting opioid leading to respiratory depression (Hardman *et al.* 1996), the system was sensitive enough to detect the rapid effect of the substance on the respiratory system of treated rats when compared to control animals. Expected effects were also noted in Beagle dogs and Cynomolgus monkeys. In rats the depression was more evident with the analysis at one (1) min interval. Indeed for rats, analysis at onset of pharmacological effects with one (1) min average revealed higher sensitivity to detect changes after remifentanil administration. The differences in statistical results between one (1) and five (5) min average highlights the importance of post-acquisition data analysis which needs to be tailored to each test article in order to capture pharmacological effects.

A statistical method of choice is an important key in this type of studies to reveal variations of the respiratory parameters. An inclusion of a control group for inter-group comparison in respiratory safety pharmacology studies should be considered due to the variability of respiratory parameters. Multiple statistical analysis strategies are acceptable in the presence of an occasion effect and the ANCOVA with baseline as covariate is considered as an appropriate statistical plan to detect the potential respiratory changes induced by test articles (Vickers 2001).

The objective of these studies was reached. The use of the FlexiWare system could be more cost-effective and more time-efficient. It represents a major advantage over existing methods of evaluation. It allows evaluating various parameters in one operation.

The use of the FlexiWare® System should be considered as a system of choice to use in rats, dogs and cynomolgus monkeys in combination with a specific restraint unit. Dose levels that were used reliably induce bronchoconstriction, bronchodilation and central respiratory depression given the well-characterized positive control drugs that were selected. However, more studies will be needed, to compare results obtained with other methods with those obtained in this study and to determine the data collection method in order to be able to obtain a complete picture of the effects of new drugs on the respiratory system.

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