*Int. J. Morphol.,*
*41(1)*:65-72, 2023.

# Structure and Function Adaption of Lung in *Phrynocephalus vlangalii* by Multiple Mechanisms

## Estructura y Adaptación Funcional del Pulmón en *Phrynocephalus vlangalii* por Múltiples Mecanismos

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**AN, F. & ZHANG, Y.** Structure and function adaption of lung in *Phrynocephalus vlangalii* by multiple mechanisms. *Int. J. Morphol., 41(1)*:65-72, 2023.

**SUMMARY:** Respiration and water-liquid transportation are controlled by many factors in the lung. The aim of this study was to explore the structure and proteins expression in lungs of *Phrynocephalus vlangalii* by means of gross anatomy, light microscope observation, scanning electron microscope and immunohistochemistry. Results show that there were many alveoli in the lung and the walls of alveoli and capillaries were very thin. The inner surface of the lung was divided into many cystic chambers by reticular diaphragm, and the network of pulmonary capillaries was dense. Immunohistochemistry showed that AQP1 was mainly expressed in the epithelium of interstitial bronchi, parabronchiole endothelium, capillary endothelium and alveolar epithelial cells. VIP positive nerve fibers are mainly distributed in trachea, bronchial smooth muscle layer, the walls of pulmonary vessels and bronchial vessels and around submucosal glands. CECR2 is distributed in peripheral capillaries and small. Investigations of structure and proteins biology could be relevant with the adaptive strategy to drought and hypoxia environment in *Phrynocephalus vlangalii*.

**KEY WORDS: *Phrynocephalus vlangalii*; AQP1; VIP; CECR2; Immunohistochemistry.**

## INTRODUCTION

*Phrynocephalus vlangalii* is one of the special species in Qinghai-Tibet Plateau (Jin *et al*., 2007). Along with low oxygen and drought conditions during their life, the organism try to keep its species attributes unchanged and they have become accustomed to the changing environmental conditions in characteristics at the same time. But efficient metabolism in terrestrial animals usually needs high- quality energy supply, so natural selection endows the species with plasticity, which makes the structure and function of an organism suitable for its survival and continuation under specific environmental conditions (Romanes, 1888). As an important structural organ of terrestrial animals, lungs play an important role in inhaling oxygen, discharging carbon dioxide and regulating liquid transportation throughout the body (Berend, 1984). Hypoxia and water shortage are unfavorable factors for the normal development of lung function (Tuder *et al*., 2007).

As the guardian of life, the physiological activities of the lung are controlled by many factors. In normal lung tissues, airway hydration, mucosal secretion and alveolar fluid production are all related to the permeability of airway epithelial cell membrane and endothelial cell membrane. While the specific expression of various proteins contributes to the normal development and maintenance of lung function. AQPs are proteins that allow the transcellular water transportation driven by osmotic forces. AQPs are classified into essentially permeable to water and the permeability to water also maintain intracellular and extracellular fluid balance (Lanaspa *et al*., 2010). When the lung was damaged, the decrease of AQP1 expression hindered the fluid reabsorption in the pulmonary interstitial edema tissue, then resulting in a large amount of fluid accumulation among alveoli, pulmonary interstitium and capillaries, which will cause or aggravate pulmonary edema. The expression of AQP1 will gradually increase when pulmonary edema gradually improved (Wang *et al*., 2020). Vasoactive intestinal peptide (VIP) is also one of the most important neuropeptides in the lung, which has anti-inflammatory, anti-injury and cell

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This work was funded by the Educational and Teaching Reform Research Project of Lanzhou University(JYXM-2020-20417).

Received: 2022-10-24 Accepted: 2022-12-13

protection effects. When the airway epithelium is damaged by ozone stress, the expression of VIP in animal lung tissue first increases and then decreases. VIP is highly expressed when asthma and acute lung injury occur, which may be the stress reaction of the body and thus slowing down the damage of the disease to the body (Fan *et al*., 2017). Alveolar macrophages are not affected by VIP at rest, but when macrophages are activated and immune inflammation is induced, VIP can inhibit the activation of alveolar macrophages, control the inflammatory reaction in the lung and protect lung parenchyma cells from inflammatory damage (Ran *et al*., 2015). CECR2 was first found in the Cat's eye syndrome (Dawe *et al*., 2011) and expressed in the developing myoblasts and spinal cord, suggesting that this gene may also be related to myogenesis. Numerous chromosomal regions genetically linked with susceptibility or resistance to pulmonary adenomas have been described in mice using inbred strains showing widely different susceptibilities to formation of both spontaneous and chemical induced lung tumors (Fairbridge *et al*., 2010). CECR2 as an candidate gene is responsible for susceptibility or resistance to lung cancer (Lemon *et al*.,2002).

To sustain physiological homeostasis, animals may adjusts its body and have its adaptive evolution characteristics. This study through gross anatomy, light microscope observation, scanning electron microscope and immunohistochemistry methods to study the tissue structure and protein expression of lung in *Phrynocephalus vlangalii*, which is of great significance to reveal its characteristics in drought environment and for further study in its physiological function.

## MATERIAL AND METHOD

**Animals.** The present study was carried out on *Phrynocephalus vlangalii* whose body lengths measured 50mm or more. Animals were randomly selected during September to November from the GeErMu, QingHai Province, China.

**Tissues preparation for SEM.** Tissues were fixed using a mixture of 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.05M cacodylatebuffer(pH7.4). Subsequently, tissues were fixed in a mixture of 1.6 % K4FeCN6 and 2 % $OsO_4$ . After fixing, tissue sections were dehydrated in series of alcohols and acetate solutions, critical-point dried, and prepared to examination in SEM S3400 using standard procedures.

**Tissues preparation and Immunohistochemistry.** Tissues obtained from animals were fixed with 4 % paraformaldehyde for no less than 24 h, then the tissues were thoroughly washed in PBS, dehydrated in graded ethanol and embedded in paraffin. Five micrometer-thick sections were cut from each tissue. Tissue sections were deparaffinized in xylene, washed in alcohol and rehydrated in PBS. Antigen retrieval was performed in a microwave oven in 0.01M PBS (pH7.4) for 15 min, then the sections were cooled at room temperature and washed again in PBS. Endogenous peroxidase was blocked by using 3 % hydrogen peroxide for 30 min. After washing in PBS three times, the goat serum(10 %) was used for 20 min to avoid any non-specific reactions. Then, the primary antibody polyclonal rabbit anti-AQP1, VIP, CECR2 was applied (sigma, dilution 1:500) and incubated in a moist chamber at 4 ºC overnight. Sections were incubated with biotin-labelled secondary antibodies and avidin-HRP third antibodies, positive staining was detected using DAB. The sections were counterstained with hematoxylin. Negative control sections were subject to the same procedure with the exception of omitting the primary antibody.

**Analysis.** A light microscopy was utilized for the histology studies of the sections (Zeiss, Germany), and photomicrographs were recorded with a digital camera (Germany).

## RESULTS

Gross anatomy and light microscope The lungs of *Phrynocephalus vlangalii* are spindle-shaped sacs which located on both sides of the chest. In the front, it is connected with the inner surface of the body cavity through serosa and the rear end extends to the digestive system. Each bronchus enters its lung on the medial side from the apex. The outer wall of the lung is thin in gross anatomy. At low magnification, the lung is a cystic structure surrounded by thin walls (Fig. 1.A). There are abundant blood vessels (Fig. 1.B), tubular structures and alveoli on the inner surface. There is a small amount of hyaline cartilage in the outer membrane (Fig. 1.C,D), which is composed of smooth muscle and connective tissue (Fig. 1.E). Alveolus is the main part of gas exchange (Fig. 1.F), with a large number, which increases the efficiency of gas exchange. The alveoli are surrounded by abundant capillaries and elastic fibers. The alveolar wall and capillary wall are very thin, only one layer of epithelial cells, which are beneficial to the gas exchange between alveoli and blood.

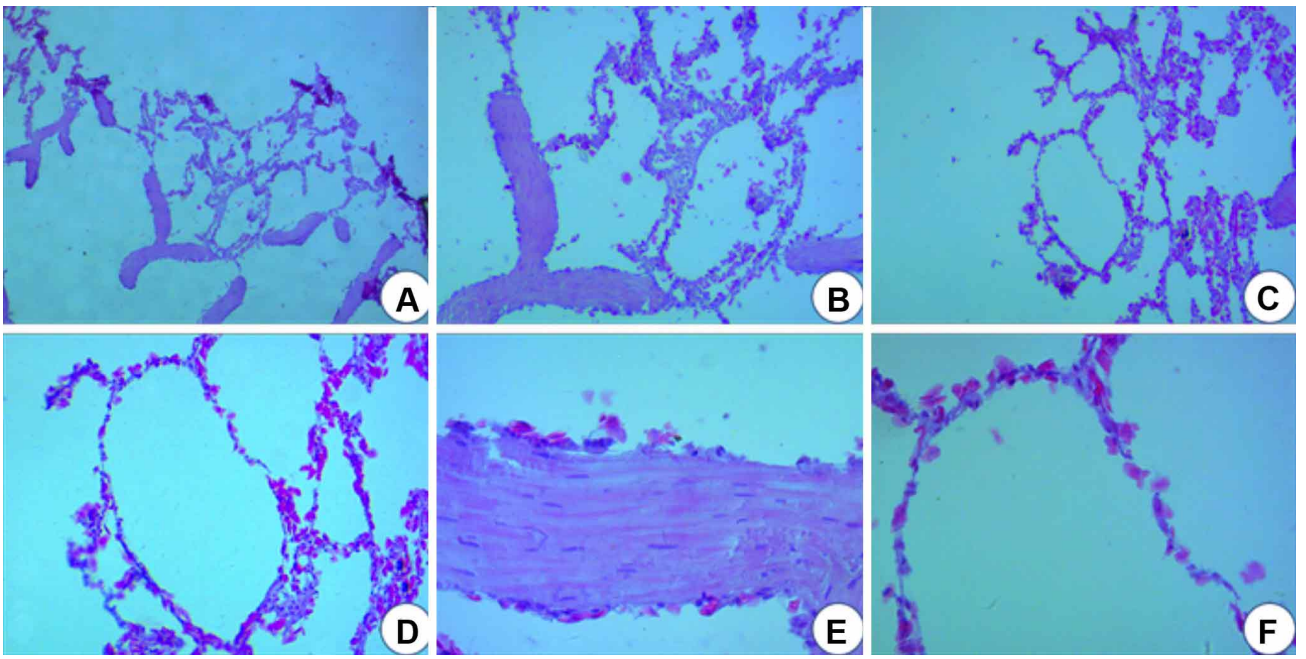


Fig. 1 Histology and microstructure of the lungs from *Phrynocephalus vlangalii*. (A)-(C)Representative images of haematoxylin and eosin of the lung. (D)Lumen of pulmonary capillary. (E)Smooth muscle beneath the connective tissue in the respiratory bronchiole. (F) Type I pneumonocyte and type II pneumonocyte in the alveoli.

**Scanning electron microscope.** The outer wall of the lung is thin and the interior of the lung contains numerous thin septa (Fig. 2.A). These septa are attached to the lung wall and their free ends serve to delineate the margins of the axial air channel (Fig. 2.B-D). On the basis of their length, the septa can be classified into three groups and the longest septa divide the internal surface of the lung into polygonal recesses(Fig. 2.B-D). The intermediate septa subdivide these recesses into smaller areas, each of which is further subdivided by the shortest septa into shallow depressions, which referred to as 'air sacs'. The cores of the septa consist of collagenous, elastic tissue together with lesser amounts of smooth muscle fibers (Fig. 2.E-F).

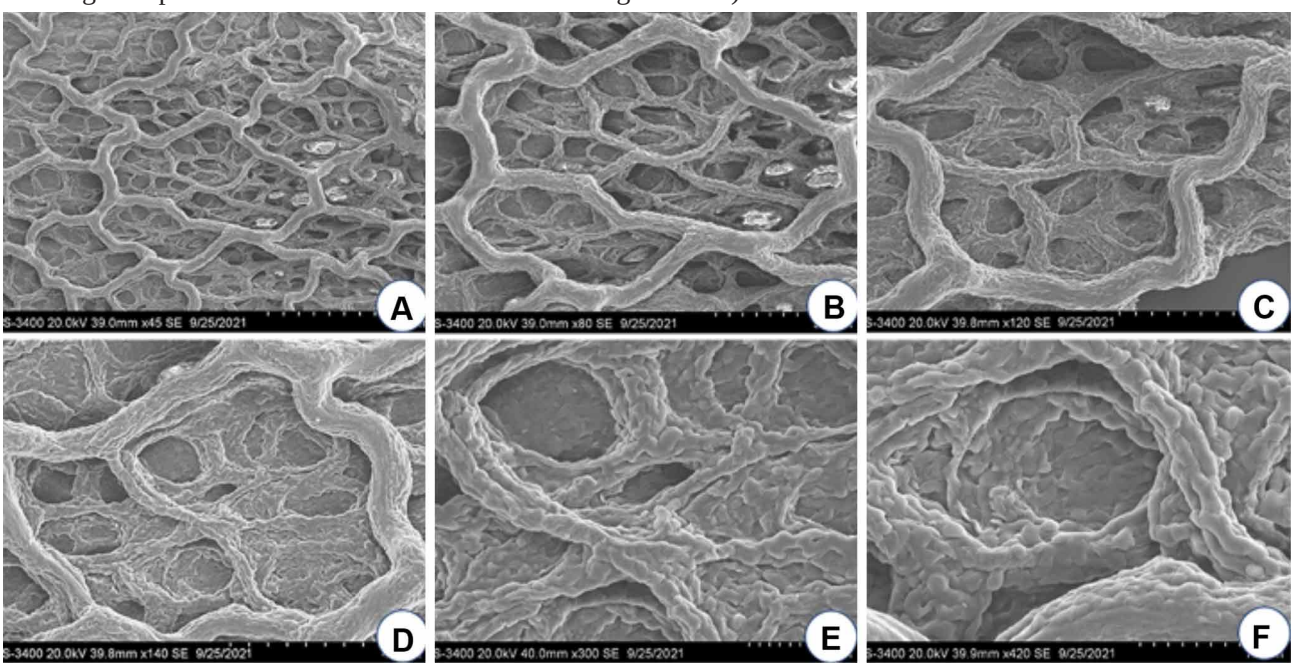


Fig. 2 Scanning electron microscopy images of the lungs from *Phrynocephalus vlangalii*. (A) Numerous thin septa in the lung. (B-D) The aveoli, primary septa, secondary septain the lung. (E-F) Collagenous, elastic tissue together with lesser amounts of smooth muscle in the lung.

**Immunohistochemistry.** In lung, strong positive reactions of AQP1 was detected in the epithelium of pulmonary interstitial bronchi (Fig. 3.A,B), the endothelium of capillaries (Fig. 3.C), the endothelium of venules (Fig. 3.D) and the epithelial cells of pulmonary alveoli (Fig. 3.E).

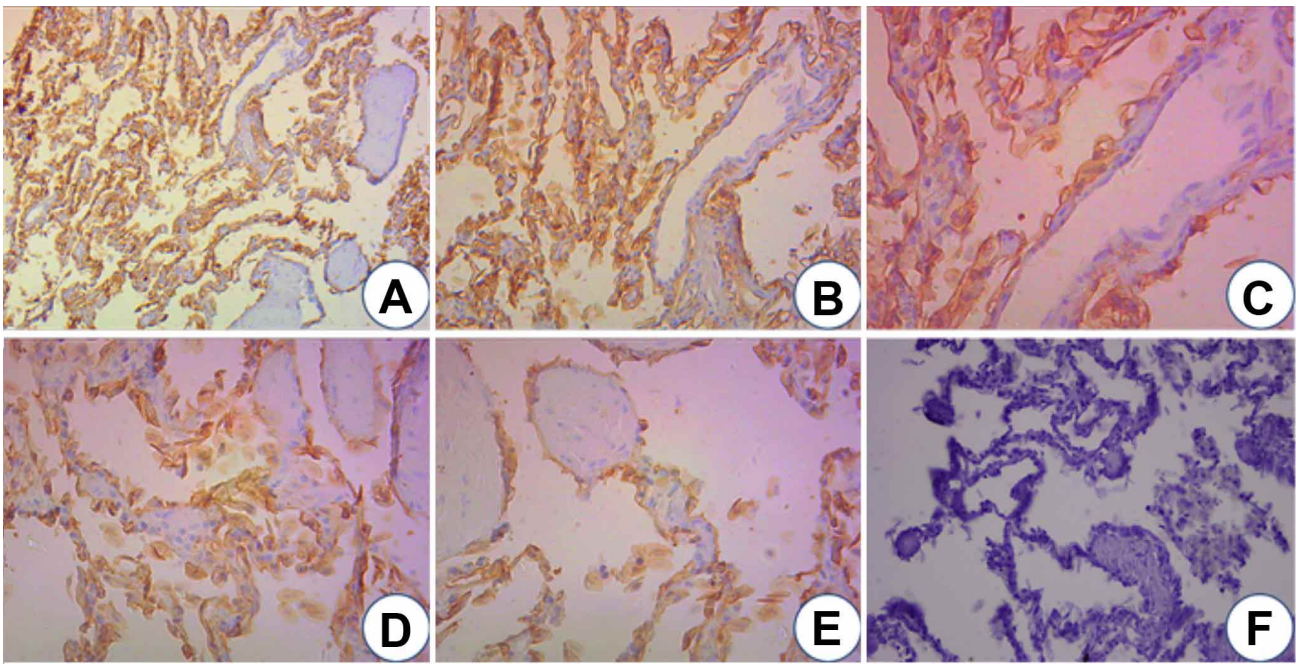


Fig. 3. Immunohistochemistry of AQP1 in the lung of *Phrynocephalus vlangalii*. (A)-(C) Immunostaining for AQP1 on the epithelial cells. (D) AQP1 expression in the venules. (E) Localization of AQP1 in the capillary and alveolar epithelial cells. (F)The negative control, in which primary antibodies were omitted.

Vasoactive intestinal peptide positive neuron are mainly distributed in smooth muscle layer of trachea (Fig. 4.A,B), in smooth muscle layer of bronchial (Fig. 4.C), in the wall of pulmonary vessel(Fig. 4.D), in the wall of bronchial vessels and around submucosal glands (Fig. 4.E).

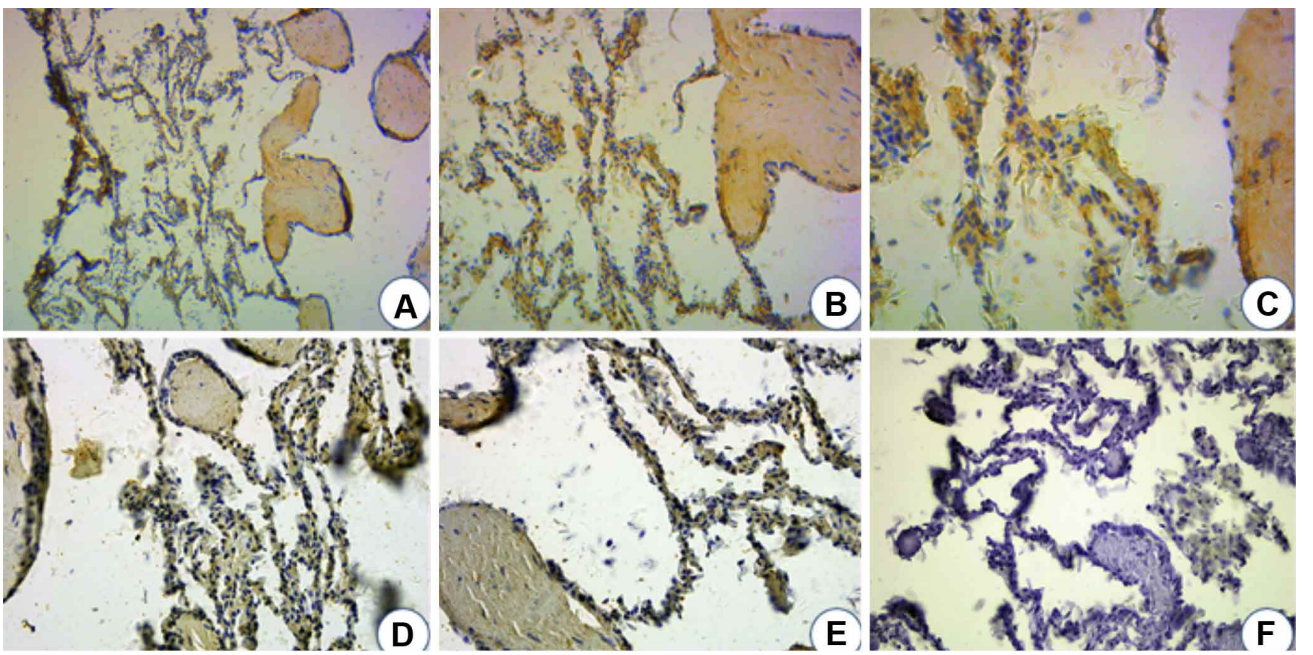


Fig. 4. Immunohistochemistry of VIP in the lung of *Phrynocephalus vlangalii*. (A)-(C) Immunostaining for VIP on the epithelial cells. (D) VIP expression in the venules. (E) Localization of VIP in the capillary and alveolar epithelial cells. (F) The negative control, in which primary antibodies were omitted.

In lung, strong positive reactions of CECR2 was detected in the alveolar septum (Fig. 5.A), alveolar wall (Fig. 5.B), the wall of respiratory bronchiole (Fig. 5.C) , the wall of bronchus (Fig. 5.D) and the wall of pulmonary vascular (Fig. 5.E).

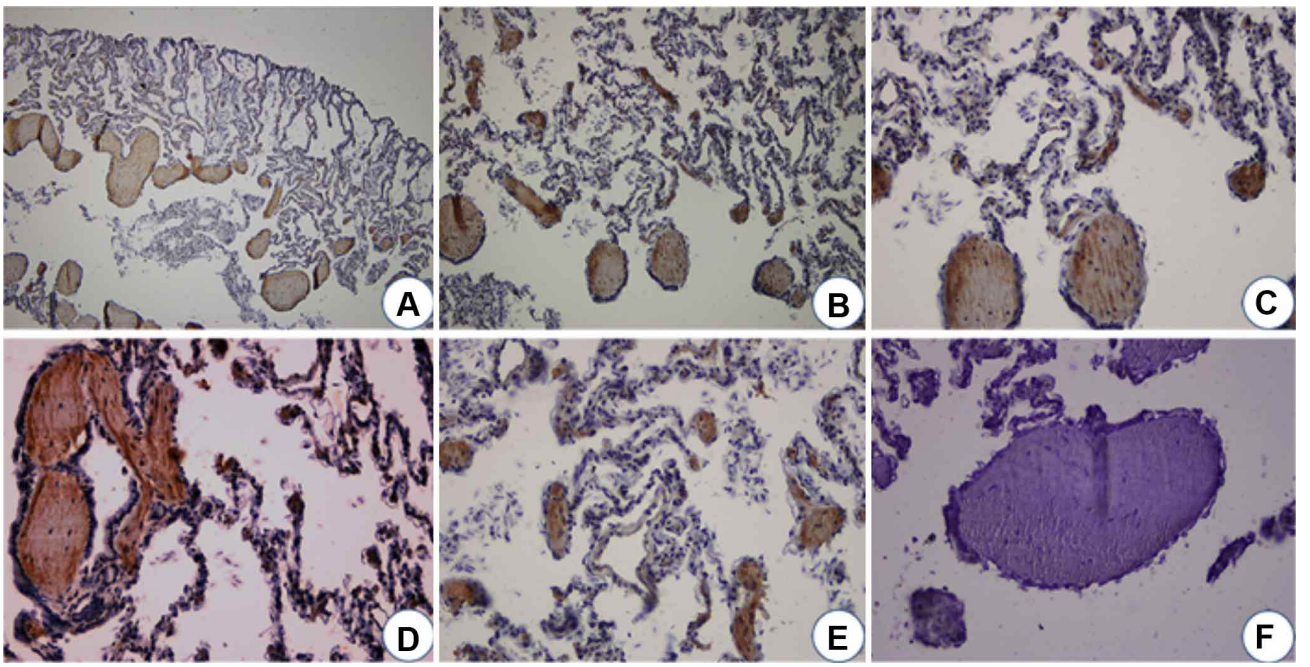


Fig. 5 Immunohistochemistry of CECR2 in the lung of *Phrynocephalus vlangalii*. (A) In alveolar septum. (B) On the alveolar wall. (C) In the wall of respiratory bronchiole. (D) In the wall of bronchus and. (E) In the wall of pulmonary vascular. (F) the negative control, in which primary antibodies were omitted.

## DISCUSSION

*Phrynocephalus vlangalii* is a viviparous, agamid sand lizard endemic to the north Tibetan plateau with a broad altitudinal range from 2000 m to 4600 m. They show extensive variation of morphology along altitudinal gradients (Jin *et al*., 2007). Faced with water shortage, drought, hypoxia and so on, their organism has become accustomed to the varying characteristics of the environmental conditions, Either short or long term adjustments in gas balance could be made by the respiratory system and circulatory system. Little is known of the responses of these organs in continuous adjustments. In this study, according to the anatomy and scanning electron microscope, the lungs of *Phrynocephalus vlangalii* are hollow and thin-walled sacs, with two equal lobes. There are many thin diaphragms in the lungs, which divide the inner surface of the lungs into polygonal depressions. The walls of alveoli and capillaries are very thin, which is beneficial to the gas exchange between alveoli and blood. A large number of alveoli increase the efficiency of gas exchange. Alveolar bread is surrounded by abundant vascular network and elastic fibers, which not only supports the morphological maintenance of the lung wall, but also makes the lung resist temporary dysfunction. Epithelial cells lined on the surface of alveoli wrap around the surface of capillaries composed of monolayer flat cells, forming a blood-blood barrier similar to that of mammals. Natural selection endows the organism with plasticity, and long-term morphological adaptation enables them to survive and continue under certain environmental conditions.

In physiological condition, lung governs respiration, which is the source of body fluid, thus, the lung plays an important role in body fluid metabolism. The transport and reabsorption of water in lung mainly include passive transport and active transport, both of which maintain the normal transport of water in lung. Through the spreading function of the lung, the water delivered by the spleen is further pushed and delivered to the fur of the whole body, thus nourishing the skin. Through the descending function of the lung, water and other subtle substances are released into the bladder, so as to ensure the smooth operation of water and liquid and to facilitate urination. Thereare all kinds of AQPs that exist on the surface of airway and lung, which promote the selective and rapid bidirectional movement of water (Wang *et al*., 2015, 2016). In normal lung tissue, airway hydration, mucosal secretion and alveolar fluid production are all related to the permeability of cell membrane, while

the cross-cell flow of water molecules between capillary endothelial cells and alveolar epithelial cells depends on the selective transport of water by AQP (Wittekindt & Dietl, 2019). Studies have confirmed that some diseases of water-liquid balance disorder are closely related to AQP, and AQP in normal physiological state may be involved in humidification of lung respiratory tract and absorption of liquid in alveoli (Verkman, 2007). In this study, it was found that AQP1 was mainly expressed in the epithelium of interstitial bronchi, parabronchiole endothelium, capillary endothelium and alveolar epithelial cells. Dry environment will lead to dehydration of the body, which affects the lung ventilation function. The blood-air barrier formed between alveolar epithelial cells and capillary endothelial cells is an important place for gas exchange, and the liquid balance in this structure has an extremely important influence on the gas exchange of the lungs. The expression of AQP1 is obviously helpful to the regulation of water carrying, promoting oxygen to pass through the blood gas barrier, relieving lung inflammation (Hentia *et al*., 2018). The decrease of AQP1 expression will hinder the fluid reabsorption in pulmonary interstitial edema tissue, resulting in a large amount of fluid accumulation among alveoli, pulmonary interstitium and capillaries and the expression of AQP1 will gradually increase with the gradual reduction of pulmonary edema, so active transporters in lung may be consistent with the overall concept of lung governs respiration.

Vasoactive intestinal peptide (VIP) is an important peptide in endocrine and immune regulation. It consists of 28 amino acid residues and has the functions of vasodilation, anti-inflammation, anti-injury and cell protection (Beshay *et al*., 2021). In this study, it was found that VIP positive nerve fibers were mainly distributed around trachea, bronchial smooth muscle layer, pulmonary and bronchial vessel walls and submucosal glands. The distribution of vasoactive intestinal peptide positive nerve fibers in these areas had the functions of dilating pulmonary vessels, relieving airway smooth muscle and promoting airway mucus secretion. In lung tissue, VIP not only can dilate blood vessels, but also has the functions of relaxing airway smooth muscle, inhibiting alveolar macrophage phagocytosis and T lymphocyte proliferation, reducing the release of inflammatory mediators, and promoting the injury and repair of airway epithelium, etc. When ozone stress causes airway epithelial injury, the expression of VIP in animal lung tissue increases at first and then decreases (Rassler *et al*., 2007). It is an important substance to maintain the physiological function of lung tissue and prevent and treat lung diseases (Frye *et al*., 2020). VIP has been proved to have a significant inhibitory effect on lung inflammation in clinical and experimental studies of novel coronavirus and acute lung injury (Sun *et al*., 2018).

Pulmonary vasoconstriction can increase pulmonary artery pressure during asphyxia in dogs (Bradford & Dean,1894) and this physiological significance lies in relieving the deficiency of alveolar ventilation capacity by increasing the ventilation ratio of alveoli (Von Euler & Von Liljestr, 1946). In HPV, it has been found that pulmonary vascular endothelial cells interact with smooth muscle cells through a variety of signaling pathways, causing pulmonary vasoconstriction (Kizub *et al*., 2013). At the same time, vasodilators produced by pulmonary vascular endothelial cells such as NO, ET-1, CO and so on, also act on the surrounding smooth muscle cells through paracrine or intercellular junction and are involved in regulating the occurrence of HPV and HPVR (Srau *et al*., 2012). Cecr2 was first identified in the analysis of human cat's eye syndrome and it contains 19 exons and encodes a protein of 1464 amino acids (Dawe *et al*., 2011). It was found that CECR2 was significantly expressed in developing myoblasts and spinal cord, and also distributed in developing body segments and neural tubes (Norton *et al*., 2022), suggesting that this gene is related to embryonic development and myogenesis. This study found that CECR2 was expressed in endothelial cells, bronchioles and alveoli confined to peripheral capillaries and venules. Contraction of smooth muscle causes bronchial spasm, while relaxation causes bronchial relaxation. Stimulating vagus nerve endings can make smooth muscle contract; stimulating sympathetic nerves can relax bronchial smooth muscle, and the use of adrenaline drugs in asthma patients can show this mechanism. The high expression of CECR2 in pulmonary micro vessels may be an adaptive compensation for high altitude hypoxia, which is of great significance for reducing pulmonary vascular resistance, enhancing oxygen diffusion ability and ensuring smooth blood circulation and oxygen transportation. It is proved that hypoxic pulmonary vasoconstriction is an adaptive physiological mechanism to optimize blood oxygen saturation, which can increase pulmonary vascular resistance in areas with poor pulmonary ventilation and make pulmonary blood flow to areas with good (Olson *et al*., 2006; Ariyaratnam *et al*., 2013), this is also the characteristic change of pulmonary arteriole smooth muscle cells on alveolar hypoxia (Madden *et al*., 1992).

As a species that has inhabited the Qinghai-Tibet Plateau for quite some time, morphological and physiological adaptations are essential for survival in such surroundings. Changes in the structure and function of the respiratory system, such as the loss of elasticity and retraction of blood vessels, increase of chest wall hardness, decrease of respiratory muscle strength, decrease of alveolar surface area, and pulmonary perfusion of capillaries, may all affect adequate ventilation and pulmonary gas exchange, resulting in VO2max limitation. However, in the lung of

Green iguanas, there is unidirectional flow along portions of the walls in both chambers and the hilus (Farmer, 2015a). It has been proposed to facilitate wash-out of lung gases, to reduce the cost of breathing, the rates of evaporative water loss, the rates of heat loss that are due to the enthalpy of vaporization, and to improve crypsis (Farmer, 2015b). Living environment for *Phrynocephalus vlangalii* is extremely arid and survival pressure may be promoted the evolution of animals to adapt to the environment. The unique structure of the lung might be related to the reason these animals rarely experience oxygen deficiency disease. Distribution of proteins in the lung of *Phrynocephalus vlangalii* suggesting that they may play constitutive role in maintaining water balance, water transmission and breathing. A better understanding of the basic structure and proteins expression may provide novel opportunities to explore the adaptive capacity of *Phrynocephalus vlangalii* in later research.

**ACKNOWLEDGMENTS**. This work was funded by the Educational and Teaching Reform Research Project of Lanzhou University(JYXM-2020-20417).

**AN, F. & ZHANG, Y.** Adaptación de la estructura y función del pulmón en *Phrynocephalus vlangalii* por múltiples mecanismos. *Int. J. Morphol., 41(1)*:65-72, 2023.

**RESUMEN:** La respiración y el transporte de agua y líquido están controlados en el pulmón por muchos factores . El objetivo de este estudio fue explorar la estructura y la expresión de proteínas en los pulmones de *Phrynocephalus vlangalii* por medio de la anatomía macroscópica, observación con microscopio óptico, microscopio electrónico de barrido e inmunohistoquímica. Los resultados muestran que había muchos alvéolos en el pulmón y que las paredes de los alvéolos y de los capilares eran muy delgadas. La superficie interna del pulmón estaba dividida en cámaras quísticas por el diafragma reticular y se observó una densa red de capilares pulmonares. La inmunohistoquímica mostró que AQP1 se expresaba principalmente en el epitelio de los bronquios intersticiales, el endotelio parabronquial, el endotelio capilar y las células epiteliales alveolares. Las fibras nerviosas VIP positivas se distribuyen principalmente en la tráquea, la capa de músculo liso bronquial, las paredes de los vasos pulmonares y los vasos bronquiales y alrededor de las glándulas submucosas. CECR2 se distribuye en pequeño capilares periféricos. Las investigaciones de la biología de la estructura y las proteínas podrían ser relevantes con la estrategia de adaptación al entorno de sequía e hipoxia en *Phrynocephalus vlangalii*.

**PALABRAS CLAVE: *Phrynocephalus vlangalii*; AQP1; VIP; CECR2; Inmunohistoquímica.**

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