Role of Rho/Rho-kinase signaling pathways in development of bronchopulmonary dysplasia in the experimental rat model

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Institute of Pathophysiology- Experimental Medicine Unit

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CONTENTS

Abbreviations

Airway smooth muscle	(ASM)
Analysis of variance	(ANOVA)
Arachidonic acid	(AA)
Bronchopulmonary dysplasia	(BPD)
Cyclooxygenase	(COX)
Danish Myo Technology	(DMT)
Epidermal Growth Factor	(EGF)
Extracellular matrix	(ECM)
Extracellular signal-regulated kinase	(ERK)
G-protein coupled receptors	(GPCRs)
Gram	(g)
GTPase-activating proteins	(GAPs)
Guanine dissociation inhibitors	(GDI)
Guanine exchange factors	(GEFs)
Guanine threephosphate	(GTP)
Insulin-like growth factor-1	(IGF-1)
Intraperitoneal	(i.p.)
Krebs Henseleit	(KH)
MAPK/ERK	(MEK)
Methacholine	(MCh)
Mitogen-activated protein kinase	(MAPK)
Myosin light chain kinase	(MLCK)
Myosin light chain phosphatase	(MLCP)
Myosin light chain	(MLC)
Nitric oxide synthase	(NOS)

Nitric oxide	(NO)
Platelet-derived growth factor	(PDGF)
Prostaglandin F2α	(PGF2α)
Receptor tyrosine kinases	(RTKs)
Rho-associated protein kinase	(ROCK)
Rho-binding domain	(RBD)
Sarcoplasmic reticulum	(SR)
Standard error of mean	(SEM)
Tracheal smooth muscle	(TSM)

1. INTRODUCTION

1.1. Structure and development of lungs

The respiratory system has a principal function to deliver the oxygen to the blood then through the blood it will be transported up to the final destinations in tissues and cells (1). The respiratory tract begins at the nose and mouth proceeding along the airways and terminates to the lungs. Among the airways the trachea is the largest one, which at the lower level is bifurcated into two smaller airways – named right and left bronchi. These structures lead to the lungs. The most distal airways, branches smaller than bronchi (bronchioles) are the narrowest airways, and these contain over 25000 distinct branching terminations. Because, the airways look like an upside-down tree, this section of the respiratory tract frequently is named the tracheobronchial tree (2).

In term of function, the system comprises of two areas. One of those named as respiratory area, which presents the site of gas exchange, is constituted of the microscopic components such as respiratory bronchioles, alveolar ducts and alveoli. All the rest of the respiratory passageways are involved in the conducting (3).

The cartilage as fibrous, semi-flexible connective tissue structures hold open the large airways. The lung tissue surrounds the small airways, sustain, and is connected to them. The airways size can be changed by circular airway smooth muscle (ASM), depending on the constricted or relaxed state they are situated (3). The mechanical properties of ASM are fundamental to the regulation of airway contractility and airway tone in vivo (4, 5). Descending to the lower levels of passageways (airways) as they become smaller, the relative amount of smooth muscle in the tube walls increases. A total layer of circular smooth muscle in the bronchioles and the deficit of supporting cartilage enables the bronchioles under certain conditions to exert a considerable resistance to air passage (3).

In the terminal part of each bronchiole are located small air sacs called alveoli, and each bronchiole ends on thousands of them. Lungs of the normal grown-ups contain approximately 300 – 400 millions of these porous, air-filled sacs that are encircled by capillaries (2). The very thin barrier that separates air and capillaries enables oxygen to move from the alveoli into the blood and enables carbon dioxide to move in the opposite direction (6). Thus, because the blood-air barrier is exceptionally slender and is penetrable to numerous gases, such as oxygen and carbon dioxide among those, the inspired air is conveyed into closeness to the circulating

blood (6). Therefore, due to these properties of the blood-air barrier gas exchange is facilitated and fulfills the Fick's law request for as narrow as possible a diffusion space (1).

The lungs are covered by the pleura which is a serous layer that overlay the inner part of thorax. The pleura enables lungs' movement along the breathing and during the movement of the individual. In general, the space between two layers of the pleura is filled with a little quantity of lubricating liquid. By changes of size and shape of the lungs, parietal and visceral pleura pass easily over each other (3).

Neonatal lungs are not simply small versions of adult lungs. Respiratory disease occurs in a setting of incomplete development of the tracheobronchial system or the pulmonary vasculature, especially in preterm infants (7).

The process of lung development is described in five phases (embryonic, pseudoglandular, canalicular, saccular and alveolar) (1, 8). Lung development happens during the embryonic period. Lung primordium in humans is formed by the end of the first month of gestation. In the fetal period, pseudoglandular, canalicular and saccular phase of lung development follow each other in sequential way and last approximately 10-12 weeks each of them (9, 10). Lung development seen in other mammalian species has a similar path even though, the timing and the beginning of each phase is distinct based on the real length of the gestational phase and the relative level of the lung growth at birth (1, 9,11).

At the end of the first month of gestation in human embryoan evagination bud from epithelium of foregut becomes visible. This embryonic lung bud contains the pluripotent stem cells that give rise to more than 40 different cell types and more than 300 million alveoli. A series of branching tubes are divided from bud quickly in a dichotomous model. Mesenchymal tissues are occupied and interdigitated by these tubular branches. The most relevant parts of the future tracheobronchial tree are created during the stadium of branching morphogenesis (7). In regulation of lung development during embryogenesis the transcription factors play crucial role which inhibit or stimulate the expression of appropriate genes involved in this process (12, 13). Interaction between mesenchyme and the epithelium plays a critical role in lung development process. This was shown by experimental embryology, where a recombination experiment was performed. In this experiment the tracheal epithelium begins functioning when it is grown on a layer of bronchial mesenchyme, confirming that this double origin of lungs is crucial for its development (12).

The following phase – pseudoglandular, is considered very critical for the development of all conducting airways. It was shown by Kitaoka et al. (13) that by the end of the pseudoglandular phase, 20 generations of conducting airways are formed.

During the canalicular phase which lasts from 16 to 24 week of gestation, dramatic changes will occur in lung anatomy, because in this period happens pulmonary epithelium differentiation, which results with the formation of the future air-blood tissue barrier. In addition, canalization of the pulmonary tissue by capillaries and surfactant production start. In this phase, continued branching of the airways forms respiratory bronchioles that represents the first gas-exchanging sites (acini) within the tracheobronchial tree, generations (9, 10, 14).

The following phase of lung development, named saccular phase, in human species it lasts from 24th week of the gestation until near term. A huge amount of amniotic liquid is produced by the pulmonary epithelium in the border of canalicular and saccular phases (circa 25 week of gestation). Starting from this moment, clinically the maturity of the lungs is determined based on the activity of cells known as pneumocytes type II who start to produce a substance called surfactant (9). In this phase gas- exchange elements could be affected by developmental damage, and this has a consequence in structural changes of the later lung parenchyma. (10, 15).

The saccular phase is characterized also with formation of the last generation of air spaces in respiratory part of bronchial tree. In the terminal part of each respiratory tract passage smooth-walled sacculi form, covered with pneumocytes type I and II. Primary septa which are located among the sacculi are still thick, containing two capillary networks deriving from the neighboring saccule. The collagen and elastic fibers are still at low percentage and the intersticial area is very well supplied with cells. In the epithelium differentiation and development, the matrix that lies above, has a significant impact (16). The end of saccular phase in underlined with formation of the extracellular matrix in the interductal and intersaccular area by interstitial fibroblasts. Throughout this time occurs growth of the vascular tree in both, length and diameter (9).

The alveolarization act which in fact is a supplemental period of saccular formation occurs between 30 and 37 week of gestation, and is identified by the development of secondary alveolar septa. The process of alveolarization goes on at postnatal period, in all species. The development of true alveoli in lungs happens after the birth in species with short gestational periods, while in species with longer periods of gestational, saccular formation is replaced by

the creation of new alveoli prior to birth. Even though alveolarization time as a process varies between different species, the process of formation of new alveoli proceeds postnatally in all mammalians. (9, 17, 18, 19).

The lungs start their function as a gas exchange organ exclusively at birth. Preterm babies very often have health problems because their organs and systems are not developed enough. Most frequently affected are lungs, brain, immune system, and gastrointestinal tract, and present the major risk for causing persistent problems and death. In preterms the most ordinary problem is Respiratory Distress Syndrome (RDS). Premature babies have lungs which have not developed enough surfactant, which represents a protective thin layer that enables lungs' air sacs to stay open (7, 20).

Life-threatening disease can occur when the development of lungs is disturbed. This is especially significant in preterm, and term born infants, in whom disturbances to late development initiate risky respiratory failure. Bronchopulmonary Dysplasia (BPD) is the most known disease of late lung development encountered in a neonatal intensive care unit (21).

1.2. Bronchopulmonary dysplasia (BPD)

BPD previously known as chronic lung disease of infancy is a lung disorder that affects mostly preterms. Patients with BPD usually experienced mechanical ventilation in a prolonged period used for RDS treatment. BPD may also develop in premature babies who had not many symptoms of early lung disease. BPD first was described by William Northway Jr. (year 1967), and it was reviewed and updated again by him in 1990 (22). Initially BPD was attributed to the exposure to high oxygen. Subsequently, it was accepted that not only hyperoxia, but oxidative stress by itself as well, might be an element which contributes in its development (23). The main listed factors that contribute to the pathogenesis of BPD were premature birth, respiratory failure, oxygen toxicity, and barotrauma.

Etiology of BPD-lung impairment involve factors such as:

- prematurity - the lungs, particularly the air sacs, are not completely developed.
- small quantity of surfactant -a component that helps keeping tiny air sacs opened
- application of oxygen as a therapy (high oxygen concentrations can injury lung cells)

- mechanical ventilation (the pressure of air from respiratory devices, airway suctioning, application of endotracheal tube). This tube is inserted in the trachea and attached to respiratory device.

The risk of BPD rises with lower birth weight. The latest research has shown that nearly in 50% of all newborns weighing <1250 g, admitted in a UK intensive care units for newborn babies, the BPD was developed (24). Infants with body weight less than 1000 g showed high incidence to develop BPD, while they weighing 1000 to 1500 g showed an incidence of 25%.

Bronchopulmonary Dysplasia pathophysiology isn`t yet completely understood and it seems to be multifactorial. This disease is a consequence of different toxic factors that can damage small airways and by interfering with alveolarization (alveolar septation). All this leads to alveolar simplification with a decrease in the total area for gas exchange. This condition will affect the developing pulmonary vasculature as well. The development of alveolar and vascular structures of the lung are closely related, hence the damage of one structure may have a negative effect on the development of the both (25). Children with BPD may also develop pulmonary hypertension. This reflects the combined effects of hypoxia-induced vasoconstriction and remodeling of the pulmonary vasculature (26).

There are four phases in the development of BPD:

- phase 1 (1-3 days), includes hyaline membrane presence, atelectasis, vascular hyperemia and lymphatic dilatation.
- phase 2 (4-10 days), in this phase are noticed bronchial obstruction and instant reparative changes in the lungs .
- phase 3 (11-20 days); remaining alveoli suffer compensatory hypertrophy, also there is hypertrophy of bronchial-wall muscle and glands.
- phase 4 (>1 month), is characterized by the presence of emphysematous alveoli (27).

BPD prognosis- Recurrent respiratory infection such as pneumonia, bronchiolitis and respiratory sincicial virus, poor growth and pulmonary hypertension present a greater risk for babies with BPD. Hyperoxia-induced toxic effects on premature lungs represent a very important and expensive health issue, for this reason the development of proper therapies is a clinical goal of great significance (28). Although therapy with oxygen has increased survival of critically ill newborns, it has also resulted in a higher incidence of chronic lung disease -

BPD (29, 30, 31). Increased airway reactivity in childhood is strongly marked when there is a history of BPD (32, 33).

The evidence have shown that oxygen toxicity to the lungs because of its interference in the production of the reactive oxygen species (ROS) which have cytotoxic potential. The most ROS-es are superoxide and hydroxyl radicals, hydrogen peroxide, and kinetically unstable oxygen (singlet oxygen). The most important physiological source for radicals is mitochondrial respiration (34). Hyperoxia additionally stimulates the oxidative stress. The increased oxidative stress has been implicated in development of BPD in newborn.

There have been developed and are in use many animal models of BPD, like mice (35), rats (36-40), rabbits (41, 42), preterm lambs (43, 44) and preterm pigs (45, 46) displaying the characteristics of BPD.

Hyperoxic exposure of rat pups serves as useful model for studying neonatal lung injury and as an experimantal model for BPD. Hyperoxia has been shown to induce airway hyper reactivity in this model by us and others (36, 47), a pathophysiological feature of BPD. The increased contractility of intra and extrapulmonary airways was associated with decreased relaxation of ASM under *in vitro* and *in vivo* conditions (36-, 39, 47, 48). Sopi et al. (39) showed that relaxant responses were impaired in hyperoxia-exposed rat pups due to decreased level of nitric oxide (NO) production, because of reduction of L-arginine bioavailability to the nitic oxide synthase (NOS).

In normal physiological conditions in the airways there is a caliber between relaxant and contractile responses. As earlier studies showed that under hyperoxic conditions there is a disturbance of this balance dominating contraction over relaxation of ASM (38-39), it is rational that the investigations to be focused on the mechanisms leading to the hyperreactivity of ASM in order to target specific molecules in treatment of BPD.

In addition to airway hyperreactivity, a recent study revealed that hyperoxia induces airway remodeling comprising epithelial thickening, sub-epithelial fibrosis, and increased α-SMA expression (49). The increase in ASM mass is in theory enough to include the main cause of airway narrowing (50, 51) and an association of airway remodeling and airway hyperreactivity has been reported (52-55).

1.3. Rho/Rho-kinase signaling and ASM contraction

G-protein RhoA, a monomeric member of Rho-subfamily in the Ras-subfamily is an upstream activator of Rho-kinase (56, 57). Activity of RhoA is regulated by the group of three regulatory proteins known as: guanine dissociation inhibitors (GDI), guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs) (58-60).

It is well established that activation of RhoA/Rho-kinase signaling can be induced by different stimuli, like contractile agonists which act on G-protein coupled receptors (GPCRs), growth factors that act on receptor tyrosine kinases (RTKs), cytokines acting on cytokine receptors and integrin-specific extracellular matrix (ECM) proteins (61-64).

Rho-kinase, a serine/threonine kinase is one of the best characterized effectors of Rho (57). There have been identified two isoforms of Rho-associated protein kinase (ROCK): ROCK1 expressed mainly in the lung, liver, spleen, kidney and testis, while the isoform ROCK2 is mostly expressed in brain and heart (65).

Direct interaction of a C-terminal Rho-binding domain (RBD) with GTP-bound RhoA activates Rho-kinase (66, 67). Besides activation by RhoA, this could be activated by arachidonic acid also in a RhoA independent fashion (65).

A numerous of downstream targets for Rho-kinase have identified, which are related with regulation of variety of cellular functions, including contraction, cell movements, cell gene transcription, cell proliferation, growth and adhesion and cytoskeletal remodeling (56, 57).

In general, the contraction of smooth muscle, is mediated by the increase of the Ca^{2+} inside the cell which derives by the activation of Ca^{2+} channels in plasma membrane and/or release of Ca^{2+} from intracellular Ca^{2+} stores (sarcoplasmatic reticulum – SR).

Elevation of cytosolic Ca^{2+} is followed by generation of the $4Ca^{2+}$-calmodulin-myosin light chain kinase (MLCK) complex and activation of MLCK. The active form of MLCK phosphorylates the 20 kDa myosin light chain (MLC_{20}), leading to smooth muscle contraction (57, 68). In addition to the Ca^{2+}-dependent phosphorylation of MLC, this is also regulated by the enzyme MLC phosphatase (MLCP) (69, 70). The extent of MLC_{20} phosphorylation is determined by the balance between the activity of MLCK and MLCP which causes MLC-dephosphorylation (70). RhoA and its active downstream target Rho-kinase interfere in this equilibrium by phosphorylating the myosin-binding subunit of MLCP, leading to the MLCP inhibition. This results in the increase of phosphorylation of MLC and subsequently the

augmented level of contraction at fixed level of cytosolic Ca^{2+} - this state is called Ca^{2+} - sensitization (56, 71, 72). Rho-kinase can also directly phosphorylate MLC, and this phosphorylation occur in the same site phosphorylated by MLCK (Figure 1).

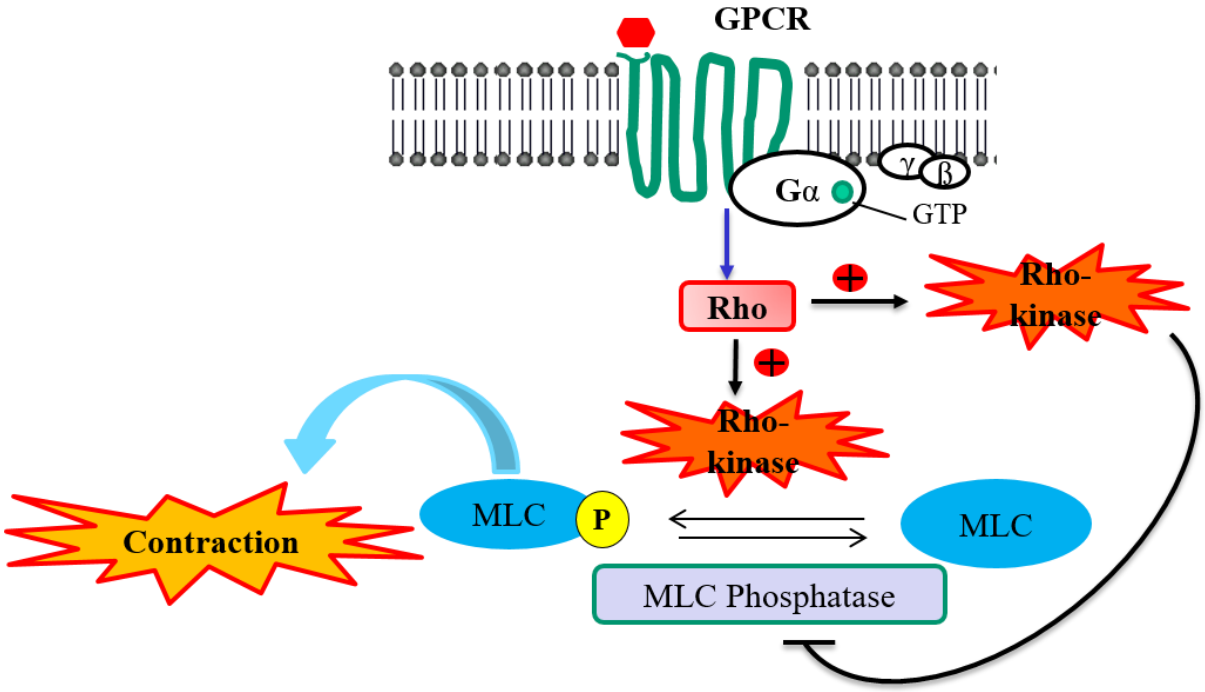

Figure 1. Rho/Rho-kinase signaling pathway

Besides the control of smooth muscle contraction under normal circumstances it has been reported for augmentation of Rho/Rho-kinase–mediated Ca^{2+}-sensitization under pathophysiological conditions (73, 74, 75). It was shown that Rho-kinase contributes to muscarinic agonists-induced contraction of bronchial smooth muscle (in rats challenged with allergen (75). Rho-kinase was demonstrated, also to be involved in airway hypersensitivity in actively sensitized guinea pigs (76). It was shown that Rho/kinase contributes in airway hyperresponsiveness to prostaglandin $F_2\alpha$ and histamine after the early and late asthmatic reaction induced by allergen and this was reversed by inhalation of Y-27632, a Rho-kinase inhibitor (77). Y-27632 suppressed the airway hyperresponsiveness in mice challenged with ovalbumin (78). These evidence suggest for important role that Rho-kinase plays in airway hyperresponsiveness under specific conditions. Pharmacological inhibitors of Rho-kinase, such as Y-27632 and fasudil, block the activity of Rho-kinase through competition with site that

ATP binds on the enzyme, thus preventing RhoA-mediated MLC phosphatase inhibition, which results in relaxation of the smooth muscle (79, 80).

In addition to functional participation of Rho-kinase in airway hyperreactivity, the evidence showed for involvement of this molecular player in airway remodeling. A study in human ASM documented the key role of Rho-kinase in proliferation induced by growth factors (81). The proliferative effect of epidermal growth factor was diminished by Rho-kinase inhibition (82).

Although in an animal model of BPD it has it has been suggested for an involvement of Rho-kinase on airway hyperreactivity induced by hyperoxia (83), however its role is not clear yet. Therefore, the scope of this thesis is to determine the role of Rho/Rho-kinase signaling on airway hyperreactivity in animals exposed to hyperoxia and the potential use of pharmacological inhibitors to reverse the adverse effects of hyperoxia in airways.

2. HYPOTHESIS

Rho/Rho-kinase signaling is involved in airway hyperreactivity-induced by hyperoxia, while *in vitro* and/or *in vivo* inhibition of Rho/Rho kinase signaling pathway will prevent the adverse effects of hyperoxia.

3. AIMS OF THE STUDY

3.1. General aim

The study of ethio-pathogenic pathways in this animal model would clarify the mechanisms responsible for progressive dysfunction of the lungs, which may contribute to better treatment and prevention of BPD disease in patients.

3.2. Specific aims

1. To determine the effects of hyperoxia on ASM contractile- and relaxant responses toward different stimuli and involvement of Rho/Rho-kinase signaling in these processes.
2. To determine the effect of pharmacological inhibitors of Rho-kinase under *in vitro* and *in vivo* conditions on physiological responses.
3. To determine the involvement of other signaling pathways in Rho/Rho kinase signaling under hyperoxic condition.

4. MATERIALS AND METHODS

4.1. Animals

Rat pups (Wistar- Rattus norvegicus) were used to perform the experiments. Rat pups from two divers offspring on the forth postnatal day (P4) were mixed in random way and set to either hyperoxic (181) or room air groups (98) and exposed for seven days to hyperoxia >95% O_2 or kept in room air. Animals (mothers) were provided with water and food *ad libitum*, while a 12-h on/12-h off light cycle was maintained. The mothers were housed together with rat pups from hyperoxic groups in a Plexiglas chamber (38 liters) and exposed for 7days to continuous flow of O_2 (2 L/min). Every day mothers were rotated between room air and hyperoxic groups to protect them from a continues hyperoxic exposure. Oxygen concentration within a chamber was monitored continuously via oxygen analyzer (MiniOX-1, Ohio Medical Corporation, IL). The pups assigned to room air were kept in a commercial rat cage in room air with their mother. Pups from both sexes were included in these experiments and no differences were observed between sexes in this study. In some sets of experiments during the exposure either to hyperoxia or room air, animals daily were injected intraperitoneally (i.p.) with a Rho-kinase inhibitors such as (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632; 10 $mg \cdot kg^{-1} \cdot day^{-1}$) or fasudil (10 $mg \cdot kg^{-1} \cdot day^{-1}$). Control groups of animals received the same volume of saline as vehicle.

This study was conducted in compliance with the rules described in guidelines for use of laboratory animals, issued by Federation of Laboratory Animal Science Associations (FELASA) and the protocol was approved by Institutional Ethical Committee of the University Clinical Center of Kosovo and Medical Faculty of the University of Prishtina (ref. 2550/13. and ref. 052-23413).

4.2. Methods

4.2.1. Tissue preparation

Asphyxiation in CO_2 was used to euthanized rat pups after the exposure time on day 12 after birth (P12). The trachea was taken off and prepared without serosal connective tissue in an ice-cold oxygenated Krebs-Henseleit (KH) buffer (concentration in mM: 118.2 NaCl, 25 $NaHCO_3$,

4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, and 10% D-glucose, pH = 7.4; all obtained from Sigma-Aldrich, Germany). From each rat pup a cylindrical portion of 3 millimeters length was separated from the middle part of the trachea and transferred into the tissue-organ bath containing KH-buffer (10 ml) at 37 °C, as previously described (36).

4.2.2. In vitro measurement of contraction and relaxation of tracheal smooth muscle (TSM)

Tracheal preparations placed in tissue-organ baths were suspended among a stainless hook at the base of the organ-tissue bath and a force displacement transducer. Tracheal smooth muscle (TSM) tension was measured by the four-channel organ bath system (DMT- 750TOBS, Danish Myo Technology, Aarhus N, Denmark) connected to the Power Lab/8SP (AD Instruments Inc, CO) (Figure 2) and by interfacing with computer was monitored-recorded using Chart 7.0 software. The tension of smooth muscle was expressed in gram (g). The first load of 0.3 g was applied, after these tissues were permitted to equilibrate for 45 min in the organ baths consisting of KH-buffer (10 ml) at 37 °C. Preparations were rinsed every 15 min with KH solution during equilibration time and the solution continuously was aerosolized with a mixture of the gases 95% O_2 and 5% CO_2.

Figure 2. DMT-Tissue Organ Bath System-750TOBS used for in vitro force measurements of TSM (Danish Myo Technology, DK Arhus N, Denmark)

4.2.2.1. Contractile responses of TSM toward methacholine (MCh)

To study the effect of hyperoxia on airway reactivity the preparations obtained from hyperoxic- and room air animals were established in the organ baths, then a dose-response curve was constructed using MCh (10^{-8} – 10^{-4} M) (Sigma-Aldrich, Germany) as an exogenous constrictive agonist. The time between doses was determined until the TSM have reached the plateau.

In another set of experiments to determine the involvement of Rho/Rho-kinase signaling on airway hyperreactivity induced by hyperoxia, after recording the control responses of ASM towards different doses of MCh the preparations were washed-out three times every five minutes with warmed KH solution where the TSM were relaxed to baseline and were allowed to establishes for 45 minutes. During this time preparations were incubated in one of Rho-kinase inhibitors ((+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632; 10 μM; Tocris, Bristol UK) or fasudil (10 μM; Tocris, Bristol UK)) for 30 minutes before a dose-response curve toward MCh (10^{-8} – 10^{-4} M) was recorded.

In a different batch of animals, pups were supplemented with the Rho-kinase inhibitor (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632; 10 $mg \cdot kg^{-1} \cdot day^{-1}$) or fasudil (10 $mg \cdot kg^{-1} \cdot day^{-1}$) by intraperitoneal (i.p.) injection. Control animals received same volume of saline as vehicle.

4.2.2.2. Electrical field stimulation (EFS)-induced relaxation experiments

In order to show the effect of hyperoxia on relaxant responses of TSM, preparations received from both hyperoxia- and room air-exposed animals were placed in organ baths as described above. A cumulative dose-response curve was built, after equilibration in order to detect a concentration of bethanechol that elicited about 75% of maximal response in TSM. A concentration of 100 μM bethanechol was found to be the most favorable dose to elicit 75% of maximal response. Tissues were pre-constricted using a single dose of bethanechol (100 μM; Sigma-Aldrich, Germany), then incremental EFS was applied to the pre-constricted TSM through platinum electrodes [1-20V alternating current (AC) at 50Hz] for 10 sec at 2-min intervals to induce relaxation. The relaxation of the TSM was expressed as percentage (%) of pre-constricted state for each preparation.

To determine whether inhibition of Rho-kinase can restore the relaxant responses impaired by hyperoxia, under *in vitro* conditions after recording the EFS-induced relaxant responses of pre-constricted TSM, followed by washing out, and equilibration, the preparations were incubated in Rho kinase inhibitors - Y-27632 (10 μM) or fasudil (10 μM) for 30 min, then EFS was applied.

In another set of experiments in order to study the effect of Rho-kinase inhibitors, pups were supplemented with the Rho-kinase inhibitor (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632; 10 $mg \cdot kg^{-1} \cdot day^{-1}$) or fasudil (10 $mg \cdot kg^{-1} \cdot day^{-1}$) by i.p. injection. Control animals received same volume of saline as vehicle. Tracheal cylinders obtained from these animals were placed in organ baths and EFS was applied to induce relaxation of pre-constricted TSM.

4.2.2.3. Epidermal Growth Factor (EGF)- and prostaglandin F2α (PGF2α)-induced contraction of TSM experiments

To investigate the involvement of growth factors and contractile prostaglandins on hyperoxia-induced airway hyperreactivity and the role of Rho/Rho-kinase signaling pathway in this cascade, the tracheal cylinders obtained from hyperoxic and room air animals after equilibration time subsequently were pre-constricted with 40 mM KCl. Following three wash-outs, maximal relaxation was established by the addition of 1 μM isoproterenol (Tocris, Bristol UK). Tissues were equilibrated at resting tension about 0.3 g and rinsed with fresh KH-buffer. Cumulative doses of EGF (0.1 – 30 ng/ml; human recombinant animal component free; Sigma-Aldrich, Germany) were applied and a dose-response curve was built. As tissues responded slowly to EGF, the time from one dose to the next dose was approximately 15 min. After obtaining the control responses from hyperoxic and room air animals, in another set of experiments was studied the molecular signaling pathway of EGF-induced contraction of TSM of hyperoxic animals by testing the responses toward the EGF in absence or presence of pharmacological inhibitors of Rho-kinase (Y-27632, 10 μM); or mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)-kinase (MEK) [1.4-diamino-2,3-dicyano-bis(2-aminophenylthio) butadiene, (U-0126, 10 μM); Tocris, Bristol UK] or cyclooxygenase (COX) (Indomethacin, 10 μM; Sigma-Aldrich, Germany). Every inhibitor was applied to the tissue/organ bath 30 min before EGF addition.

In a particular set of experiments, the role of contractile prostaglandin -PGF2α on Rho-kinase pathway under hyperoxic conditions was investigated. After equilibrations of tracheal preparations obtained from hyperoxic and room air animals, tissues subsequently were pre-constricted with 40 mM KCl. After three wash-outs, in order to achieve maximal relaxation preparations were treated with 1 μM isoproterenol. Tissues were equilibrated at resting tension about 0.3 g and rinsed with fresh KH-buffer every 15 minutes. A dose-response curve toward $PGF_2\alpha$ (1nM – 10 μM; Tocris, Bristol UK) in absence or presence of Rho-kinase inhibitors (Y-27632; 10 μM or Fasudil; 10 μM) which were applied 30 min before PGF2α was added to the tissues in tissue/organ baths.

4.2.3. Statistical analysis

Data are presented as mean ± SEM. Two-way ANOVA was used to determine the statistical significance, with repeated measurements to determine the differences between MCh-; EFS-; EGF- or PGF2α-induced contraction or relaxation responses of hyperoxia vs room air; hyperoxia treated vs. hyperoxia untreated; room air treated vs. room air untreated groups.

To analyze the variations between individual concentrations of particular drug or individual voltages, post hoc comparison via Tukey-Kramer multiple comparison test was used. $p<0.05$ was considered statistically significant, in all cases.

5. RESULTS

5.1. Effects of hyperoxia in contraction of ASM

Hyperoxia overall significantly increased contractile responses of ASM toward MCh compared with contractile responses generated in preparations obtained from room air-exposed animals (*P < 0.01*). As shown in Figure 3, the contractile responses of TSM from pups exposed to hyperoxia (n = 10) were significantly greater than those from TSM of room air pups (n = 10) at concentrations $10^{-6.5}$ – 10^{-4} M of MCh. The maximal values of contractile responses of TSM in hyperoxia- and room air-exposed groups were 1.8 ± 0.17 g and 1.13 ± 0.15 g, respectively.

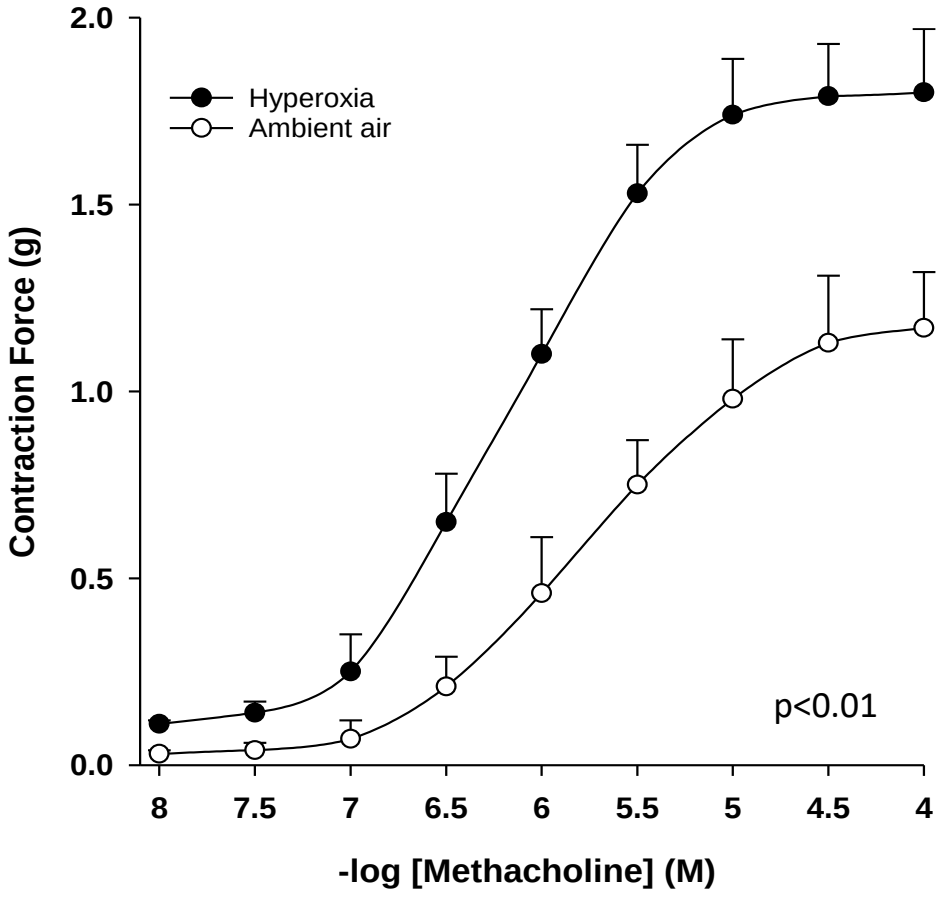

Figure 3. Effect of hyperoxia on contractile responses of TSM of rat pups. *In hyperoxic group of animals (n = 10) contractile responses were greater than in room air group of animals (n = 10). (Hyperoxia vs. Room air; P < 0.01). Data represent mean ± SEM of 10 experiments for each condition, and each performed in duplicate.*

5.2. Role of Rho-kinase on hyperoxia-induced airway hyperreactivity

5.2.1. Effect of *in vitro* inhibition of endogenous Rho-kinase activity on MCh-induced contraction of TSM

In vitro inhibition of Rho-kinase activity reversed the hyperoxia-induced airway hyperreactivity toward MCh. The contractile responses of ASM were significantly (*P < 0.001*) decreased when the preparations were pre-incubated in Rho-kinase inhibitors – Y-27632 or fasudil as compared to the responses in absence of these inhibitors (Figures 4 and 5; n = 10, in both sets of experiments).

As shown in Figure 4, when preparations of hyperoxic animals were pre-incubated in Y-27632, the contractile responses significantly decreased at concentrations $10^{-6.5}$ – 10^{-4} M of MCh as compared to control responses of the TSM from same animals in absence of this inhibitor (overall, P < 0.001). Inhibition of Rho-kinase activity reversed contractile responses of TSM to normal level, and the maximal values of contractile responses of TSM in hyperoxia + Y-27632 and hyperoxia control groups were 1.79 ± 0.17 g and 1.05 ± 0.16 g, respectively.

Also, *in vitro* inhibition of Rho-kinase activity using fasudil as inhibitor, significantly (*P < 0.001*) decreased contractile responses of TSM as compared to the responses of hyperoxic TSM obtained in absence fasudil (overall, P < 0.001, Figure 5). Inhibition of Rho-kinase activity reversed contractile responses of TSM almost to the normal level, and the maximal values of contractile responses of TSM in hyperoxia control and hyperoxia + fasudil groups were 1.86 ± 0.13 g and 1.35 ± 0.12 g, respectively.

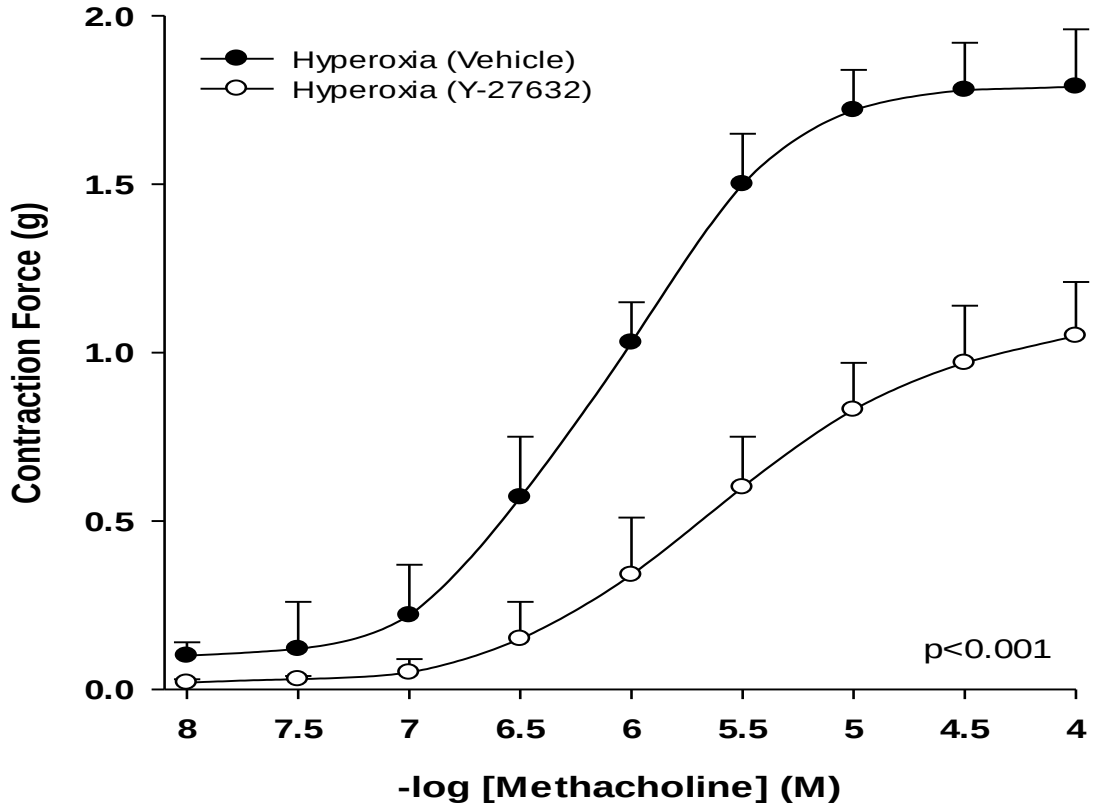

Figure 4. Effect of in vitro Rho-kinase inhibition (by Y-27632) on contraction of TSM induced by MCh in hyperoxia-exposed rat pups.

In presence of Rho-kinase inhibitor (Y-27632, 10 μM) contractile responses of hyperoxic TSM were lower than those recorded in absence of this inhibitor (n = 10) (Hyperoxia + Y-27632 vs. H + vehicle; P < 0.001). Data represent mean ± SEM of 10 experiments for each condition, and each performed in duplicate.

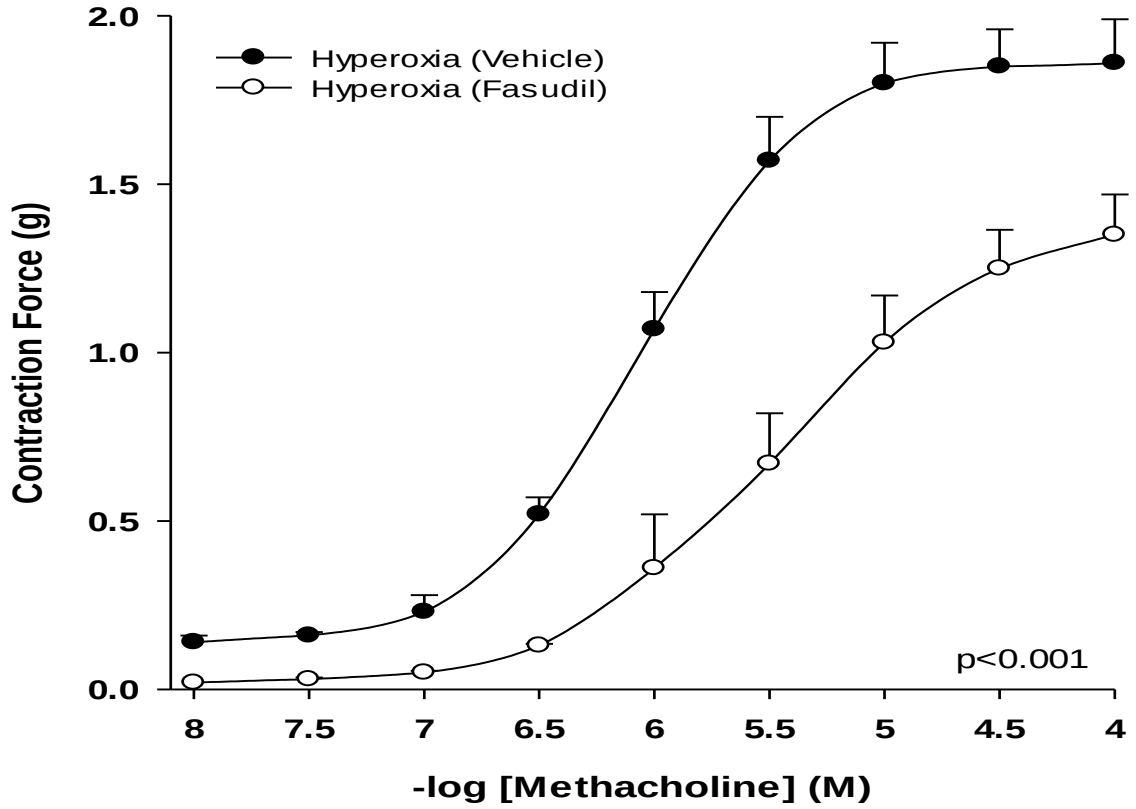

Figure 5. Effect of in vitro Rho-kinase inhibition (by fasudil) on contraction of TSM induced by MCh in hyperoxia-exposed rat pups.

In presence of Rho-kinase inhibitor (fasudil, 10 μM) contractile responses of hyperoxic TSM were lower than those recorded in absence of this inhibitor (n = 10) (Hyperoxia + fasudil vs. H + vehicle; P < 0.001). Data represent mean ± SEM of 10 experiments, and each performed in duplicate.

In vitro inhibition of Rho-kinase activity by Y-27632 or fasudil did not made a significant change on MCh-induced contraction of TSM in room air group of rat pups (n = 12) (Figure 6). The maximal values of contractile responses obtained from room air control; room air + Y-27632 and room air + fasudil, were 1.02 ± 0.13 g; 0.96 ± 0.12 g; and 0.95 ± 0.10 g, respectively.

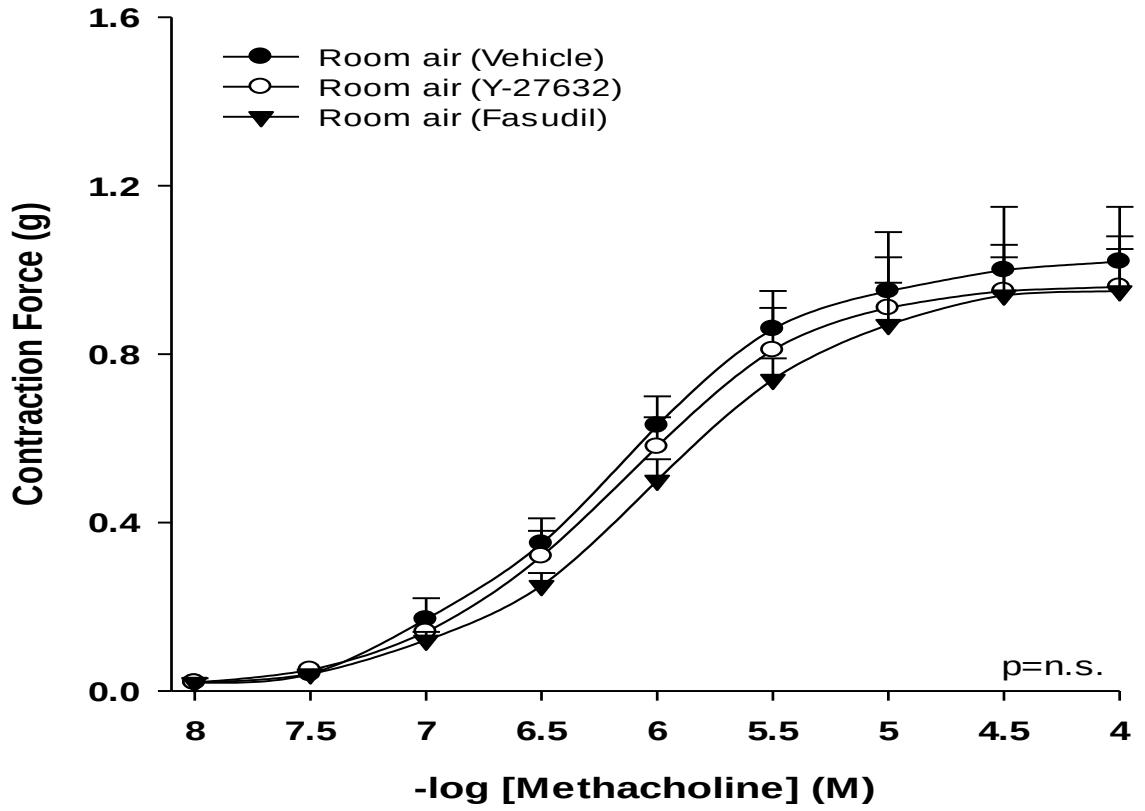

Figure 6. Effect of in vitro Rho-kinase inhibition (by Y-27632 or fasudil) on contraction of TSM induced by MCh in room air-exposed rat pups.

Presence of Rho-kinase inhibitors (Y-27632; 10 μM or fasudil, 10 μM) did not change contractile responses in room air rat pups as compared to the responses recorded in absence of inhibitors (n = 12) (Room air + Y-27632 & Room air + fasudil vs. Room air + vehicle; p = n.s). Data represent mean ± SEM of 6 experiments for each condition, and each performed in duplicate.

5.2.2. Effect of *in vivo* inhibition of endogenous Rho-kinase activity on MCh-induced contraction of TSM

In order to assess the effect of inhibition of Rho-kinase activity under whole physiological circumstances rat pups were daily supplemented with Rho-kinase inhibitors (Y-27632 or fasudil) parallel to hyperoxic or room air exposure. *In vivo* inhibition of Rho-kinase likewise *in*

vitro inhibition of Rho-kinase reversed the hyperoxia-induced airway hyperreactivity toward MCh, which overall significantly decreased (*P < 0.001*) contractile responses of TSM to MCh when compared to responses from hyperoxic control animals (overall, P < 0.001, Figure 7). In both groups supplemented either with Y-27632 (n = 8) or fasudil (n = 8) the contractile responses were restored to normal level. The difference in contractile responses significantly showed to be at concentrations $10^{-6.5}$ – 10^{-4} M of MCh as compared to the responses of the TSM from hyperoxic control group. The maximal values of contractile responses of TSM in hyperoxia control; hyperoxia + Y-27632 and hyperoxia + fasudil were 2.15 ± 0.17 g; 1.39 ± 0.16 g and 1.17 ± 0.17 g, respectively.

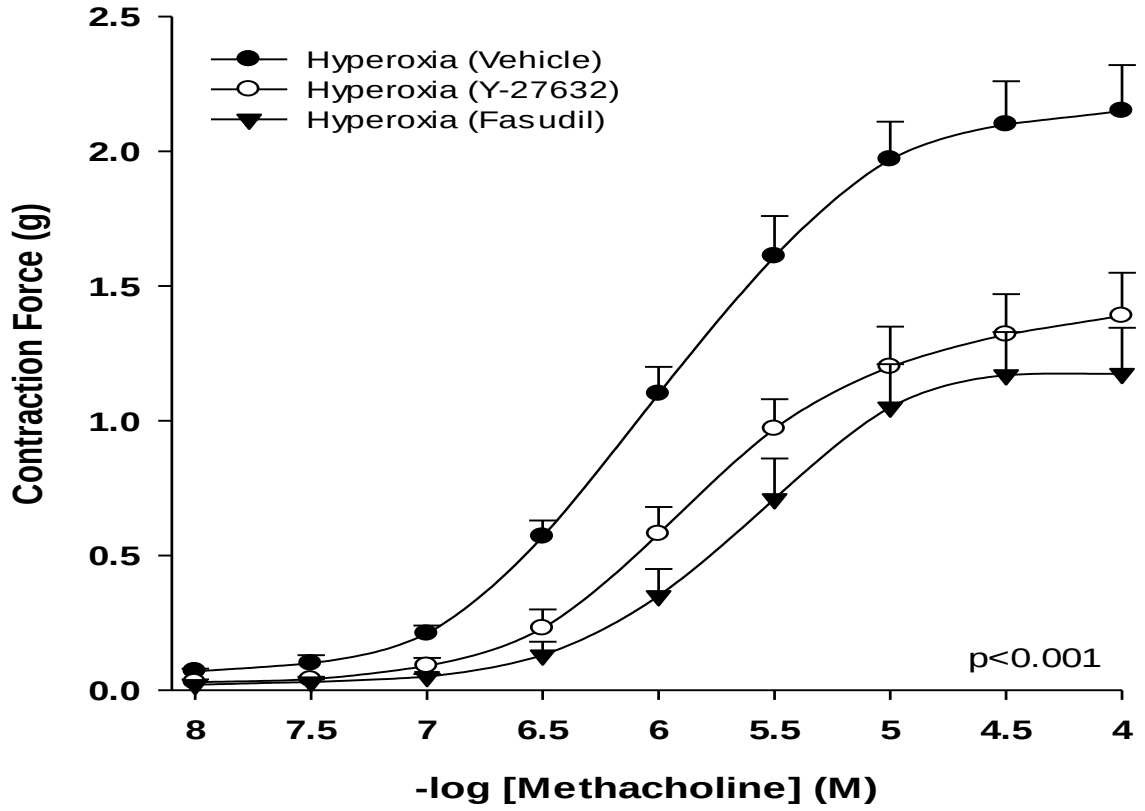

Figure 7. In vivo supplementation of rat pups with Rho-kinase inhibitor prevents development of hyperoxia-induced airway hyperreactivity toward MCh.

Supplementation of animals with Y-27632 (10 mg·kg^{-1}·day^{-1}; n = 8) or fasudil (10 mg·kg^{-1}·day^{-1}; n = 8) reversed the hyperoxia-induced increase in contractile responses of TSM toward MCh. (Hyperoxia + Y-27632 & Hyperoxia + fasudil vs. Hyperoxia + vehicle). Data represent mean ± SEM of 8 experiments for each condition, and each performed in duplicate.

In vivo supplementation of room air-exposed rat pups with Rho-kinase inhibitor (Y-27632; n = 8) or fasudil (n = 8) did not made a significant change in contractile responses compared to the control animals (vehicle) (n = 8) (Figure 8). The maximal values of contractile responses obtained from room air control; room air + Y-27632, and room air + fasudil were 1. 20 ± 0.16 g; 1.15 ± 0.15 g; and 1.12 ± 0.10 g, respectively.

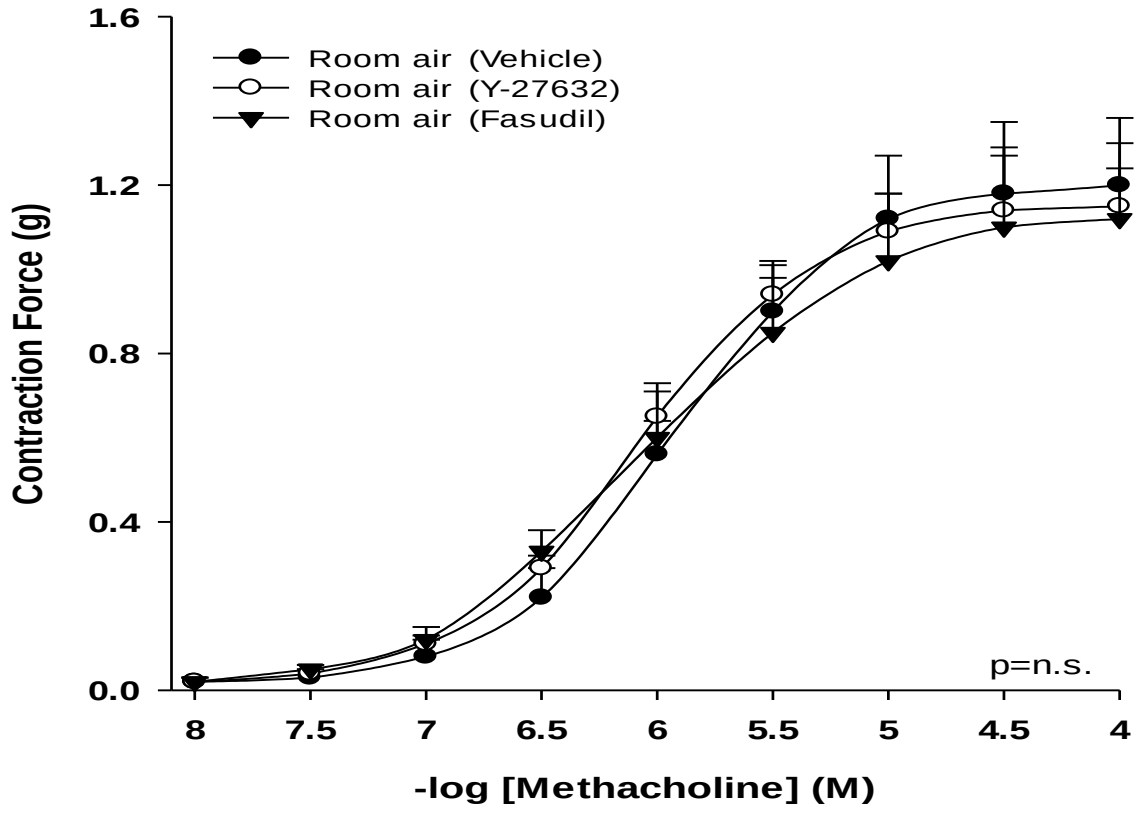

Figure 8. Effect of in vivo Rho-kinase inhibition (by Y-27632 or fasudil) on contraction of TSM induced by MCh in room air-exposed rat pups.

Supplementation of Rho-kinase inhibitors (Y-27632; or fasudil,) to the animals did not change contractile responses in room air rat pups as compared to the responses recorded from room air control group. (Room air + Y-27632 & Room air + fasudil vs. Room air + vehicle; p = n.s). Data represent mean ± SEM of 8 experiments for each condition, and each performed in duplicate.

5.3. Effect of hyperoxia in ASM relaxation

In order to study the effects of hyperoxia on relaxation of ASM, tracheal preparations obtained from hyperoxia- and room air-exposed animals were established in tissue/organ baths then pre-contracted using a single dose of bethanechol (100 μM) and incremental EFS was applied using different voltages (1 – 20V) for 1 sec each voltage every 2 min.

In hyperoxic animals, the relaxation of TSM induced by EFS overall was significantly (*P < 0.001*) reduced in comparison to rat pups exposed in room air. As shown in Figure 9, the relaxant responses of TSM from pups exposed to hyperoxia (n = 10) were significantly lower than those from TSM of room air pups (n = 10), particularly at higher volateges (8 – 20V). The data of relaxant responses in hyperoxic and room air group ranged from 0.30 ± 0.04% at 1V to 38.80 ± 4.20% at 20V, and from 0.50 ± 0.05% at 1V to 82.02 ± 6.20 % at 20V, respectively.

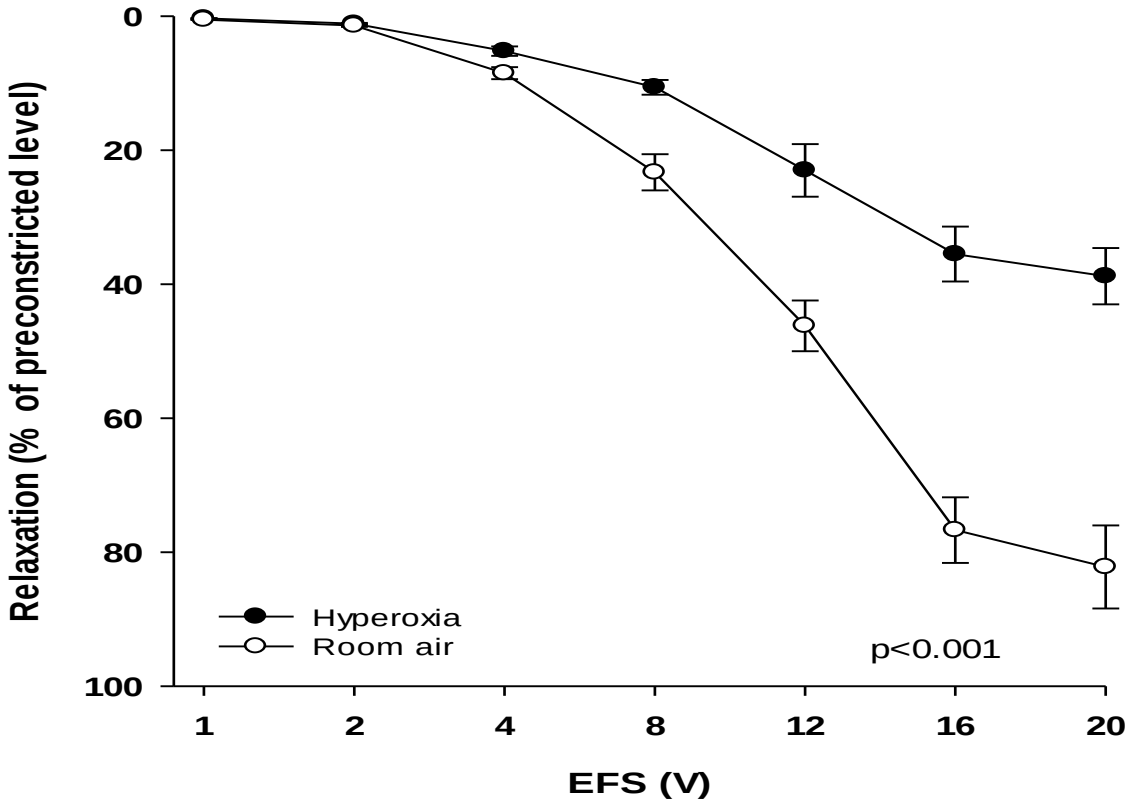

Figure 9. Effect of hyperoxia on EFS-induced relaxation of the TSM of rat pups.

EFS- induced relaxation of TSM was impaired in preparation received from hyperoxia-exposed rat pups (n = 10) as compared to those obtained from room-air exposed rat pups (n = 10). (Hyperoxia vs. Room air; P < 0.001). Data represent mean ± SEM of 10 experiments for each condition, and each performed in duplicate.

5.4. Effect of Rho-kinase inhibition on hyperoxia-induced impairment of ASM relaxation

5.4.1. Effect of *in vitro* inhibition of endogenous Rho-kinase activity on EFS-induced relaxation of TSM

In vitro inhibition of Rho-kinase activity reversed the effects of hyperoxia on relaxant responses of ASM. The relaxant responses of ASM in hyperoxic group were restored to normal level and significantly increased (overall, *P < 0.001*) when the preparations were pre-incubated in Rho-kinase inhibitors – Y-27632 or fasudil as compared to the relaxant responses in absence of these inhibitors (Figures 10 and 11; n = 10, in both sets of experiments).

As shown in Figure 10, when preparations of hyperoxic animals were pre-incubated in Y-27632, the relaxant responses significantly increased (overall, P *< 0.01*) compared to hyperoxic control responses, especially at higher voltages (8 – 20V). The data ranged from 0.60 ± 0.04% at 1V to 72.30 ± 5.60% at 20V, while in control group responses, the data varied from 0.20 ± 0.03% at 1V to 40.20 ± 3.60% at 20V.

In vitro inhibition of Rho-kinase activity using fasudil as inhibitor, also significantly (overall, *P < 0.001*) increased relaxant responses of TSM as compared to the responses of hyperoxic TSM obtained in absence of fasudil, at 4V to 20V (Figure 11). The data in hyperoxia treated with fasudil and hyperoxia vehicle ranged from 0.50 ± 0.03% at 1V to 81.20 ± 5.10% at 20V, and from 0.40 ± 0.04% at 1V to 42.10 ± 3.80% at 20V, respectively.

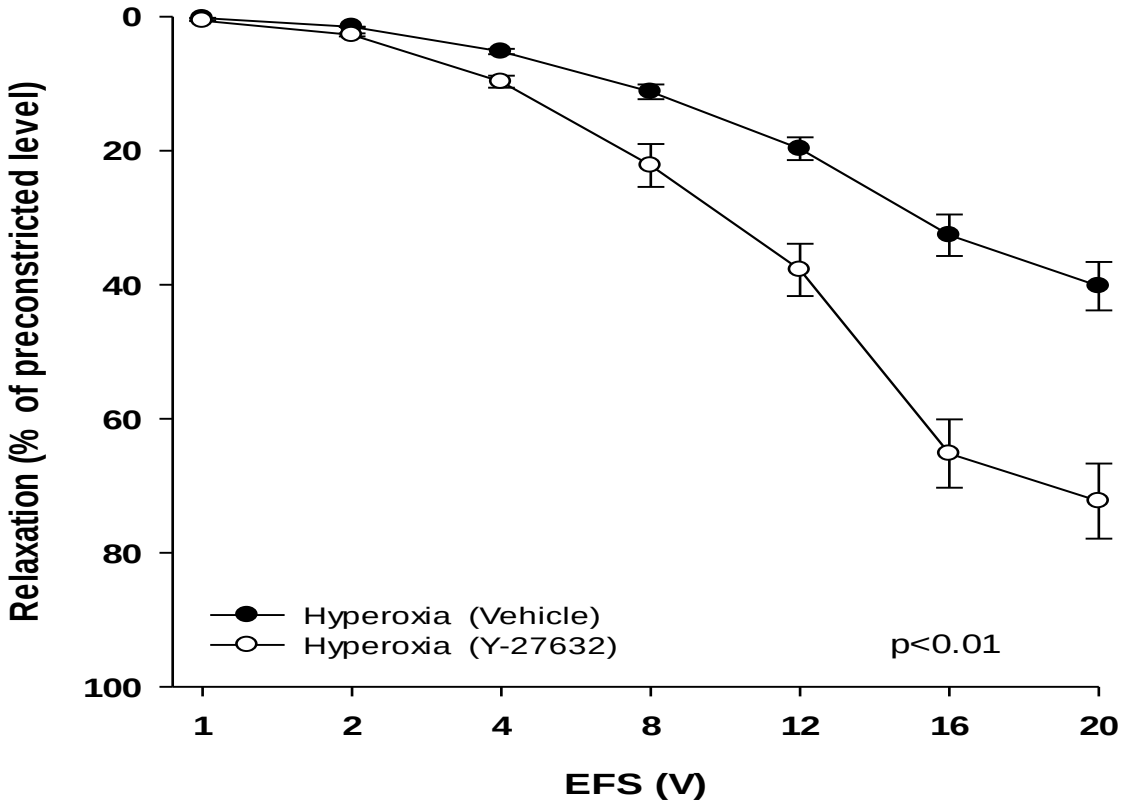

Figure 10. Effect of in vitro inhibition Rho-kinase by Y-27632 on EFS-induced relaxation of TSM in hyperoxia-exposed rat pups.

In presence of Rho-kinase inhibitor (Y-27632, 10 μM) relaxant responses of hyperoxic TSM were higher than those recorded in absence of this inhibitor (Hyperoxia + Y-27632 vs. H + vehicle; P < 0.01). Data represent mean ± SEM of 10 experiments, and each performed in duplicate.

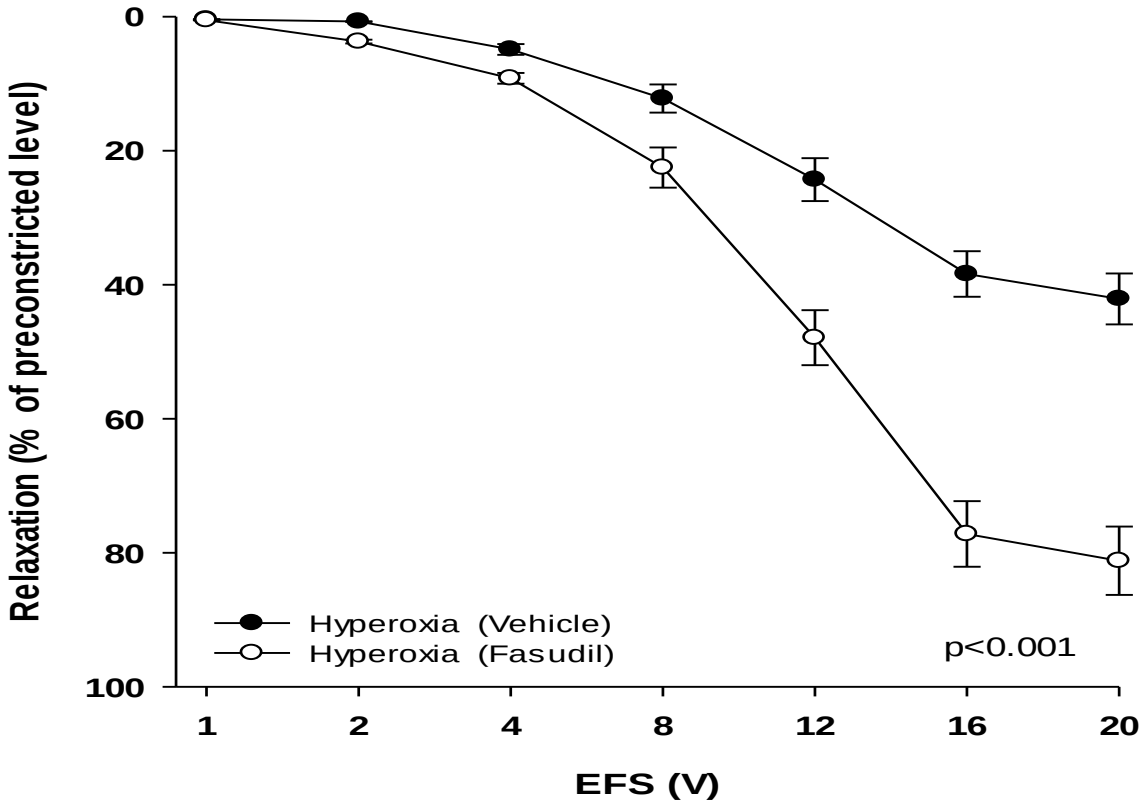

Figure 11. Effect of in vitro inhibition of Rho-kinase by fasudil on EFS-induced relaxation of TSM in hyperoxia-exposed rat pups.

In presence of Rho-kinase inhibitor (fasudil, 10 μM) relaxant responses of hyperoxic TSM were higher than those recorded in absence of this inhibitor. (Hyperoxia + fasudil vs. H + vehicle; P < 0.001). Data represent mean ± SEM of 10 experiments, and each performed in duplicate.

In vitro inhibition of Rho-kinase activity by Y-27632 or fasudil did not made any significant change in the EFS-induced relaxation of TSM in room air group of rat pups (n = 12) (Figure 12). The data obtained from room air control, room air + Y-27632 and room air + fasudil ranged from 0.30 ± 0.03% at 1V to 80.90 ± 4.90% at 20V; 0.45 ± 0.02% at 1V to 83.70 ± 4.70% at 20V, and 090 ± 0.02% at 1V to 81.70 ± 6.20% at 20V, respectively.

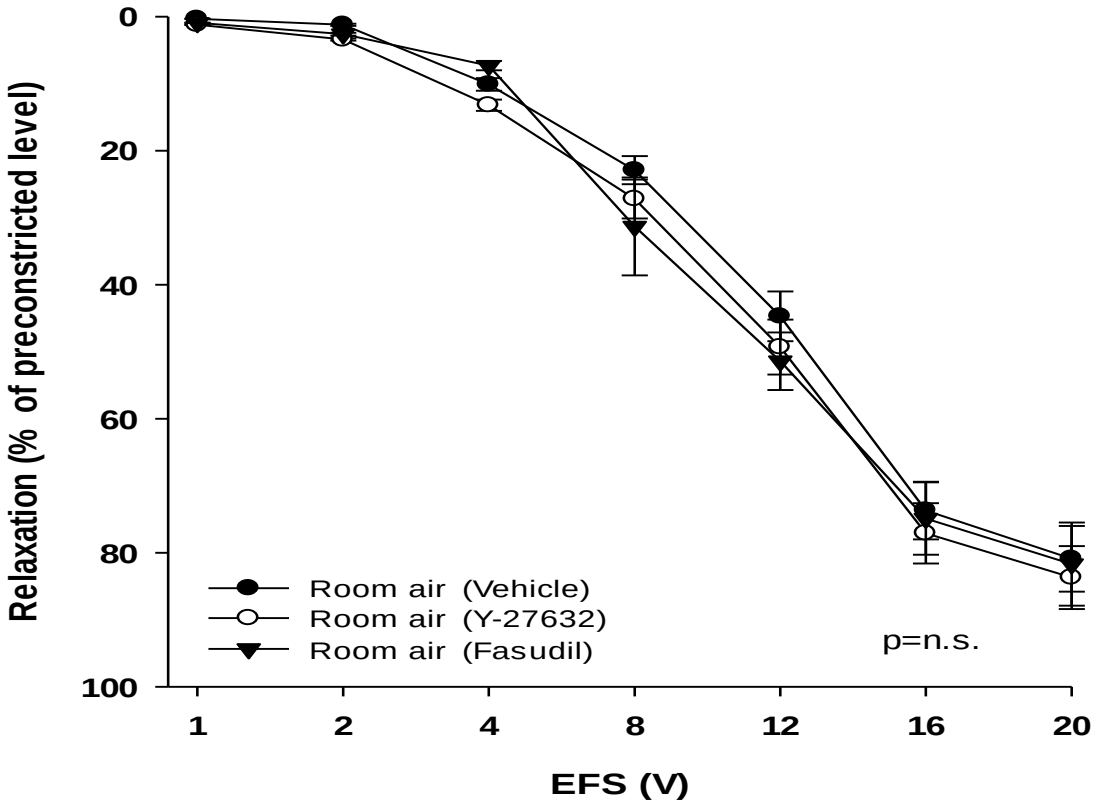

Figure 12. Effect of in vitro Rho-kinase inhibition by Y-27632 or fasudil on EFS-induced relaxation of TSM in room air-exposed rat pups.

In vitro inhibition of Rho-kinase (by Y-27632; or fasudil,) did not change relaxant responses in room air rat pups compared with control responses (Room air + Y-27632 & Room air + fasudil vs. Room air + vehicle; P = n.s). Data represent mean ± SEM of 12 experiments, and each performed in duplicate.

5.4.2. Effect of *in vivo* inhibition of endogenous Rho-kinase activity on EFS-induced relaxation of TSM

Supplementation of animals with Rho-kinase inhibitors (Y-27632 or fasudil) during hyperoxic exposure prevented the hyperoxia-induced impairment of ASM relaxation tested in tracheal preparations. Both inhibitors when supplemented to the rat pups significantly increased (overall, $P < 0.001$) EFS-induced relaxation of TSM of animals exposed to hyperoxia (Figures 13 and 14).

When Y-27632 was supplemented in hyperoxic animals the relaxant responses were significantly greater (*P < 0.001*) compared to relaxant responses from hyperoxic control animals (n = 10, each group) and these relaxant responses returned to normal level like those recorded in room air animals. The differences were significant at voltages 4 - 20V (Figure 13). The data obtained from hypeoxia + Y-27632 ranged from 0.50 ± 0.04% at 1V to 77.90 ± 3.80% at 20V; while in control group the data varied from 0.20 ± 0.03% at 1V to 37.20 ± 3.50% at 20V.

Reduced relaxation due to hyperoxic exposure was also restored when fasudil was supplemented to the animals, and these relaxant responses were significantly greater (overall, P < 0.001) as compared to control responses obtained from hyperoxic animals (n = 10, each group) (Figure 14). The differences were significant at voltages 4 - 20V (Figure 14). The data obtained from hypeoxia + fasudil ranged from 0.60 ± 0.03% at 1V to 73.90 ± 5.30% at 20V; while in control group the data varied from 0.30 ± 0.05% at 1V to 31.30 ± 2.80% at 20V.

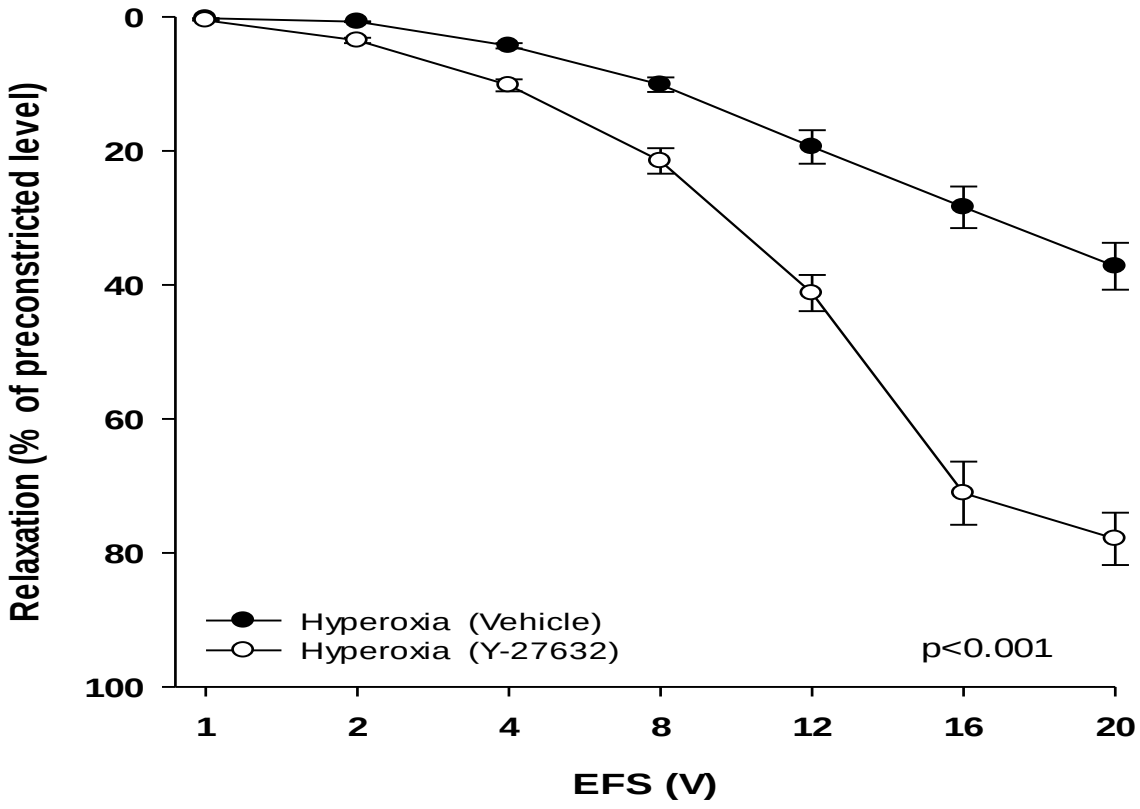

Figure 13. Effect of in vivo Rho-kinase inhibition by Y-27632 on EFS-induced relaxation of TSM in hyperoxia-exposed rat pups.

In animals supplemented with Y-27632 (10 mg·kg^{-1}·day^{-1}; n = 10) relaxant responses of hyperoxic TSM were higher than in animals un-supplemented with this inhibitor. (Hyperoxia + Y-27632 vs. H + vehicle; P < 0.001). Data represent mean ± SEM of 10 experiments, and each performed in duplicate.

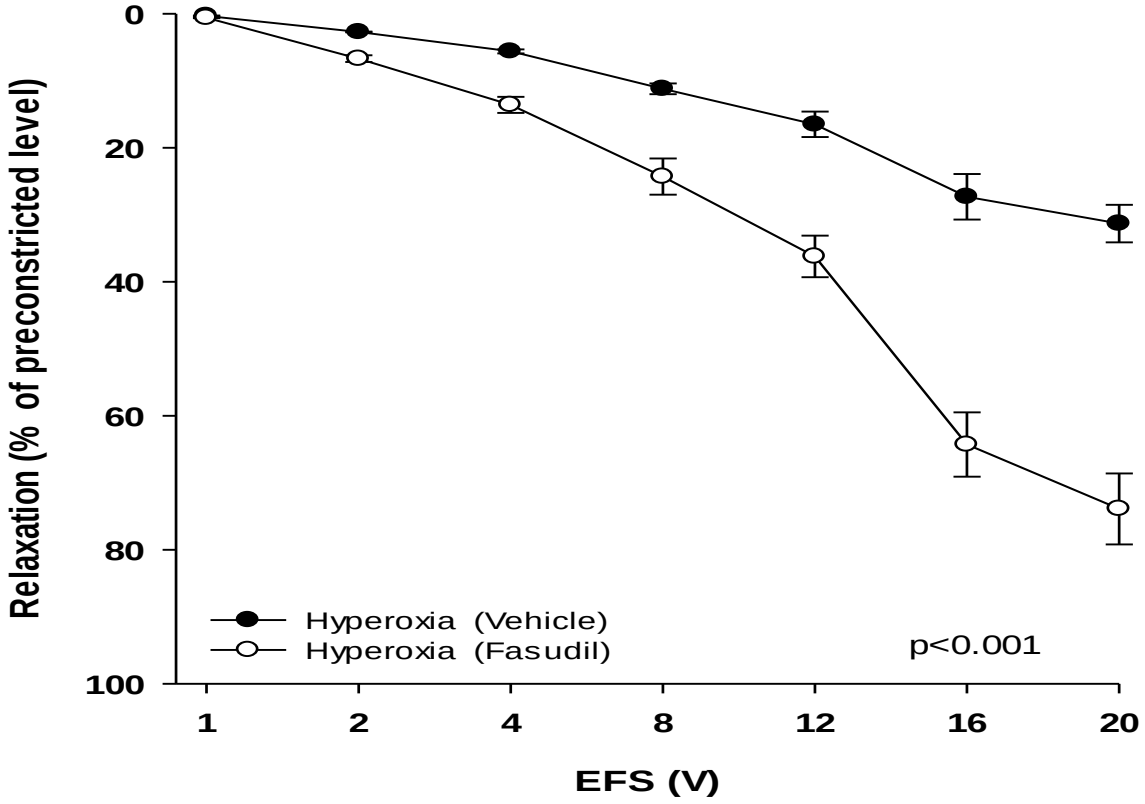

Figure 14. Effect of in vivo Rho-kinase inhibition by fasudil on EFS-induced relaxation of TSM in hyperoxia-exposed rat pups.

In animals supplemented with fasudil (10 mg·kg^{-1}·day^{-1}; n = 10) relaxant responses of TSM were higher than those recorded from control animals. (Hyperoxia + fasudil vs. H + vehicle; P < 0.001). The data are obtained from 10 individual pups per group, all performed in duplicate.

In vivo inhibition of Rho-kinase activity by Y-27632 or fasudil did not show any significant change in the EFS-induced relaxation of TSM in room air group of rat pups (n = 12) (Figure 15). The data obtained from room air control, room air + Y-27632 and room air + fasudil ranged

from 0.70 ± 0.04% at 1V to 76.10 ± 5.30% at 20V; 1.10 ± 0.01% at 1V to 74.5± 6.10% at 20V, and 090 ± 0.03% at 1V to 83.40 ± 4.90% at 20V, respectively.

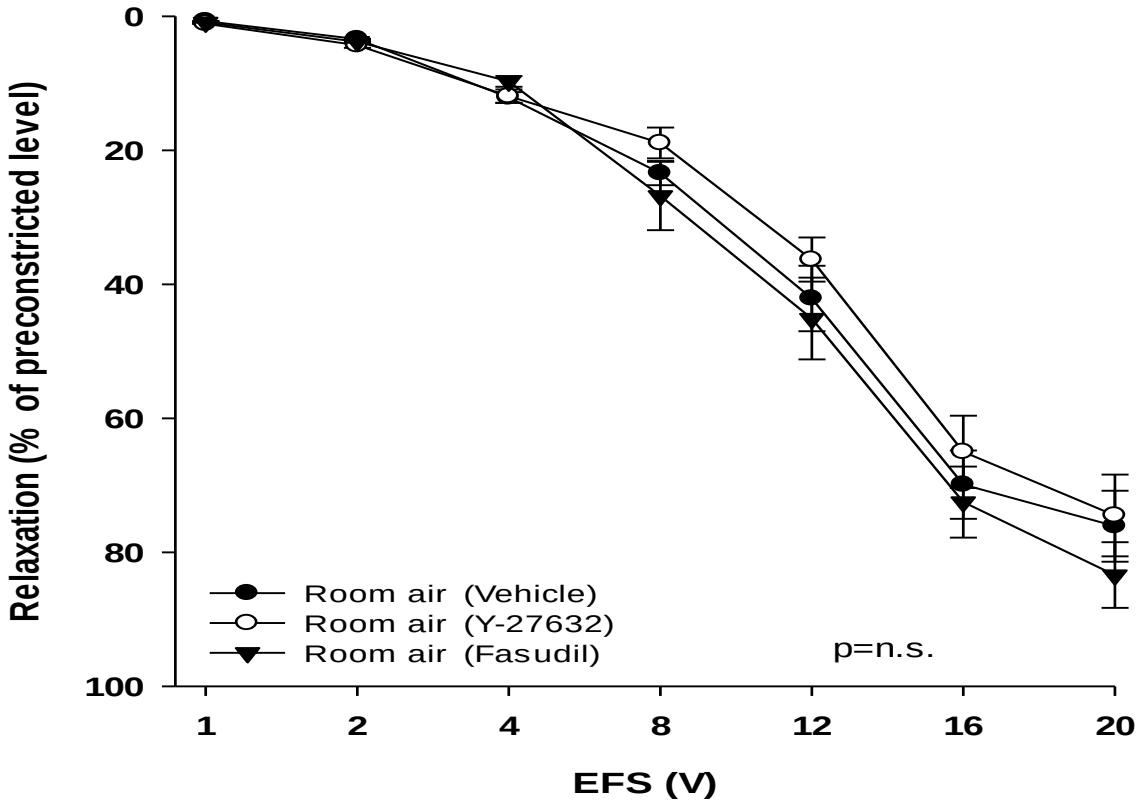

Figure 15. Effect of in vivo inhibition of Rho-kinase on EFS-induced relaxation of TSM in room air-exposed rat pups.

In vivo inhibition of Rho-kinase activity (by Y-27632; or fasudil,) did not change relaxant responses in room air rat pups compared with control responses (n = 10, each group). (Room air + Y-27632 & Room air + fasudil vs. Room air + vehicle; P = n.s). Data represent mean ± SEM of 12 experiments, and each performed in duplicate.

5.5. Role of Rho-kinase on the hyperoxia-induced increase in contraction of ASM toward EGF

5.5.1. ASM contractile responses toward EGF

In order to define the involvement of growth factors on hyperoxia-induced airway hyperrecativity the tracheal preparations obtained from hyperoxic- and room air-exposed rat pups were treated with different concentrations of EGF (0.1, 0.3, 1, 3, 10 and 30 ng/ml) and a concentration-response curve was constructed. As shown in Figure 16. in the group of hyperoxic animals the contractile responses of TSM were significantly greater ($P < 0.001$) as compared to the responses in the group of room air animals, particularly in concentrations 1-30 ng/ml (n = 8, each group). The maximal values of contractile responses of TSM in hyperoxic and room air groups were 0.58 ± 0.03 g; and 0.34 ± 0.05 g.

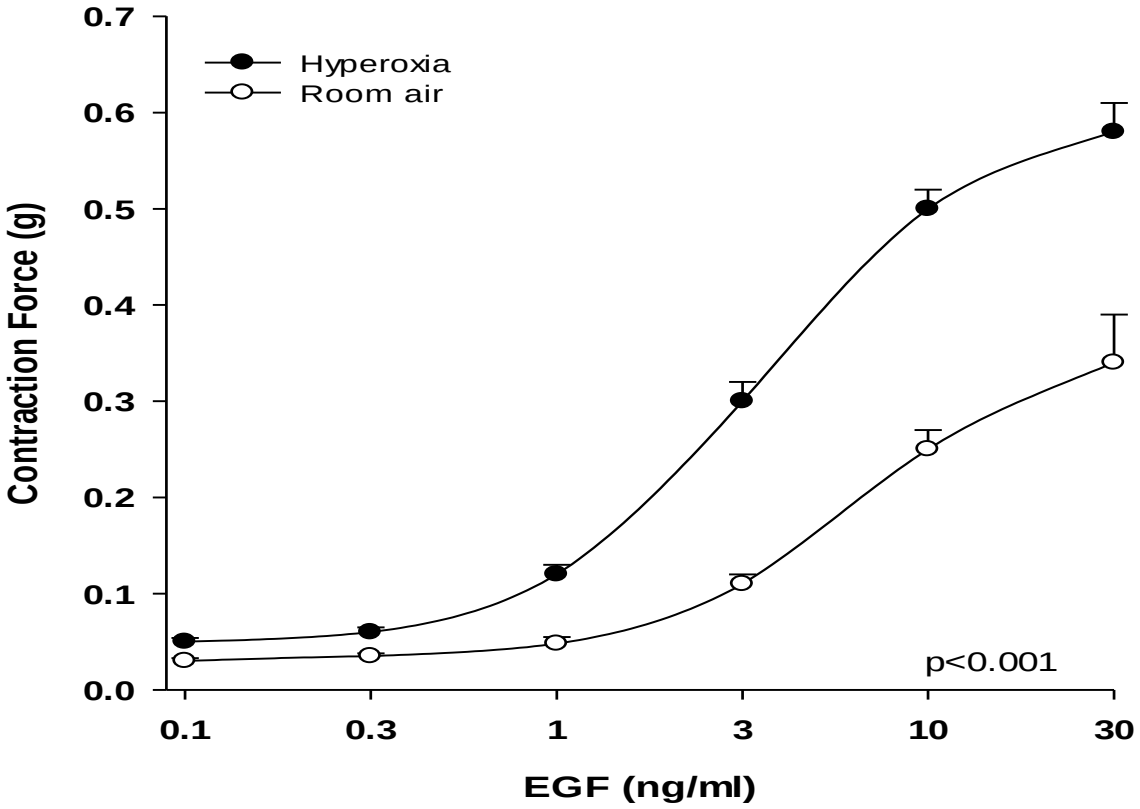

Figure 16. EGF-induced contraction of TSM preparations of rat pups.

The contractile responses were greater in hyperoxic group of animals (n = 8) than in room air group of animals (n = 8). (Hyperoxia vs. Room air; P < 0.001). Data represent mean ± SEM of 8 experiments for each condition, and each performed in duplicate.

5.5.2. Effect of inhibition of Rho-kinase, MEK and COX on EGF-induced contraction of TSM

To investigate the signaling pathway of EGF involvement in hyperoxia-induced increase of contractile responses the effects of Rho-kinase, MEK and COX inhibitors were studied. EGF-induced contractile responses were almost completely blocked in presence of Rho-kinase inhibitor (Y-27632); MAPK inhibitor (U-0126) or COX inhibitor (indomethacin) (P < 0.001; Figure 17) (n = 7, for each condition). The maximal values of contractile responses of TSM in hyperoxic and room air groups were 0.58 ± 0.03 g; and 0.34 ± 0.05 g.

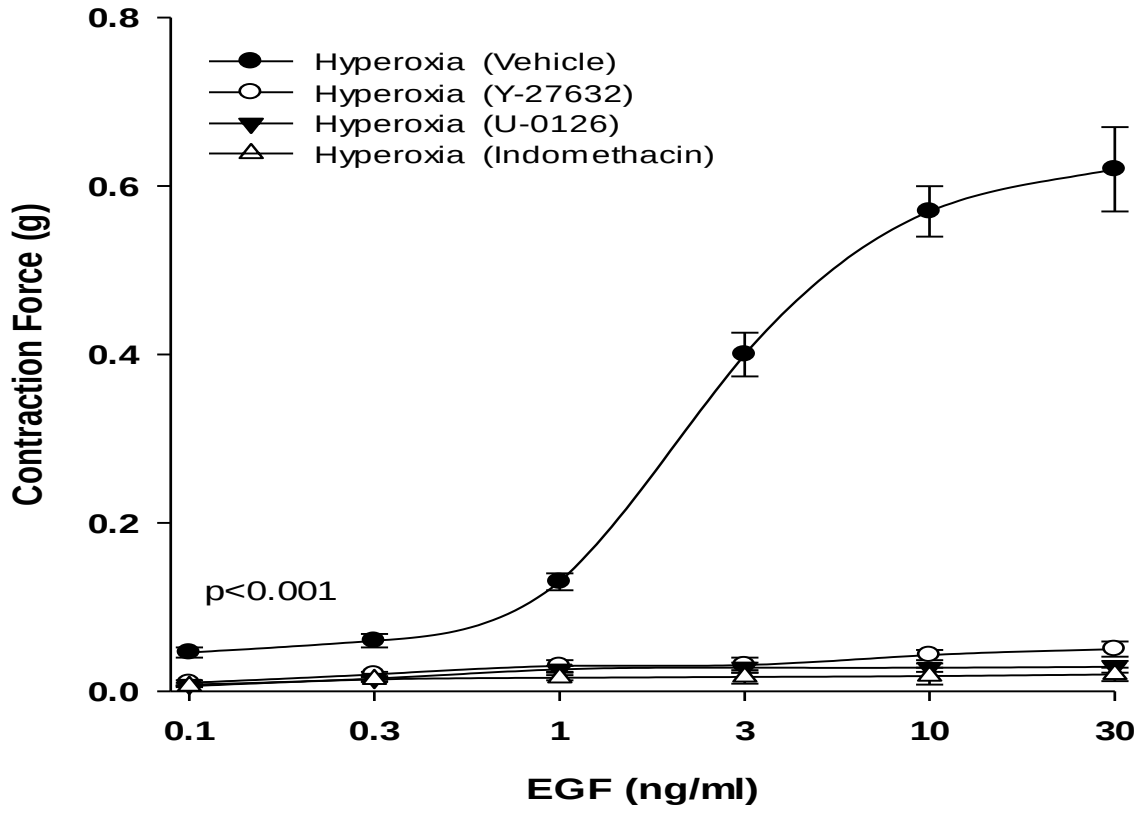

Figure 17. Effects of Y-27632, U-0126 and indomethacin on EGF-induced contraction of TSM of rat pups exposed to hyperoxia.

Use of Y-27632 (10 µM), U-0126 (10 µM) or indomethacin prevented the contractile responses of TSM toward EGF. (Hyperoxia vs. Hyperoxia + inhibitors, P < 0.001). Data represent mean ± SEM of seven separate experiments for each condition, and each performed in duplicate.

5.6. Effect of hyperoxia on $PGF_2\alpha$-induced contraction of TSM

To determine the involvement of contractile prostaglandins on hyperoxia-induced airway hyperreactivity, preparations from hyperoxia- and room air-exposed rat pups were treated with cumulative doses of $PGF_2\alpha$ (10^{-9} – 10^{-5} M). In hyperoxic group of animals (n = 8) the contractile responses were significantly greater (P < 0.001) as compared to responses obtained from room air group (n = 8) (Figure 18). The maximal values of contractile responses of TSM in hyperoxic and room air groups were 85.1 ± 5.2 (% of 40M KCl); and 54.3 ± 3.9 g (% of 40 M KCl).

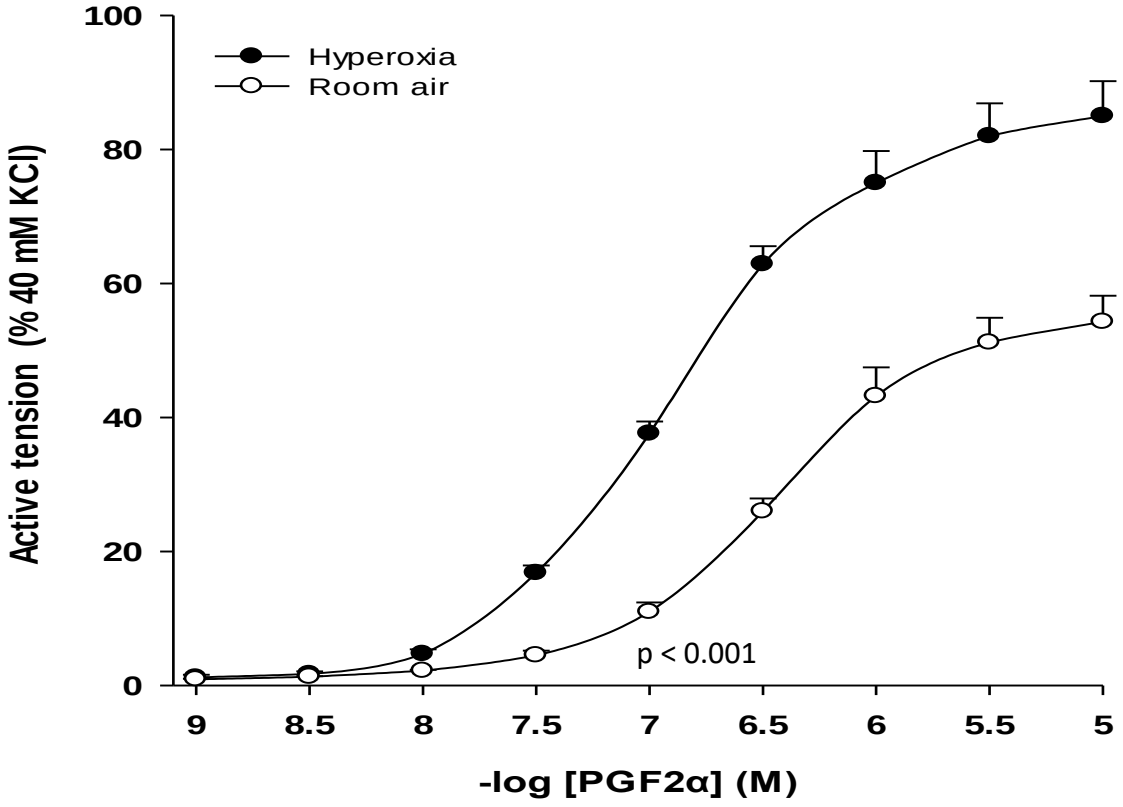

Figure 18. $PGF_2\alpha$ -induced contraction of TSM preparations of rat pups.

The contractile responses were greater in hyperoxic group of animals (n = 8) than in room air group of animals (n = 8). (Hyperoxia vs. Room air; P < 0.001). Data represent mean ± SEM of 8 separate experiments for each condition, and each performed in duplicate.

5.6.1. Role of Rho-kinase on $PGF_2\alpha$-induced contraction of TSM

To study the role of Rho-kinase on increased contractile responses in hyperoxic group toward $PGF_2\alpha$, the preparations were pre-incubated in Rho-kinase inhibitor Y-27632 or fasudil. In both cases, presence of inhibitor significantly decreased (*P < 0.001*) the $PGF_2\alpha$-induced contractile responses of TSM (Figures 19 and 20).

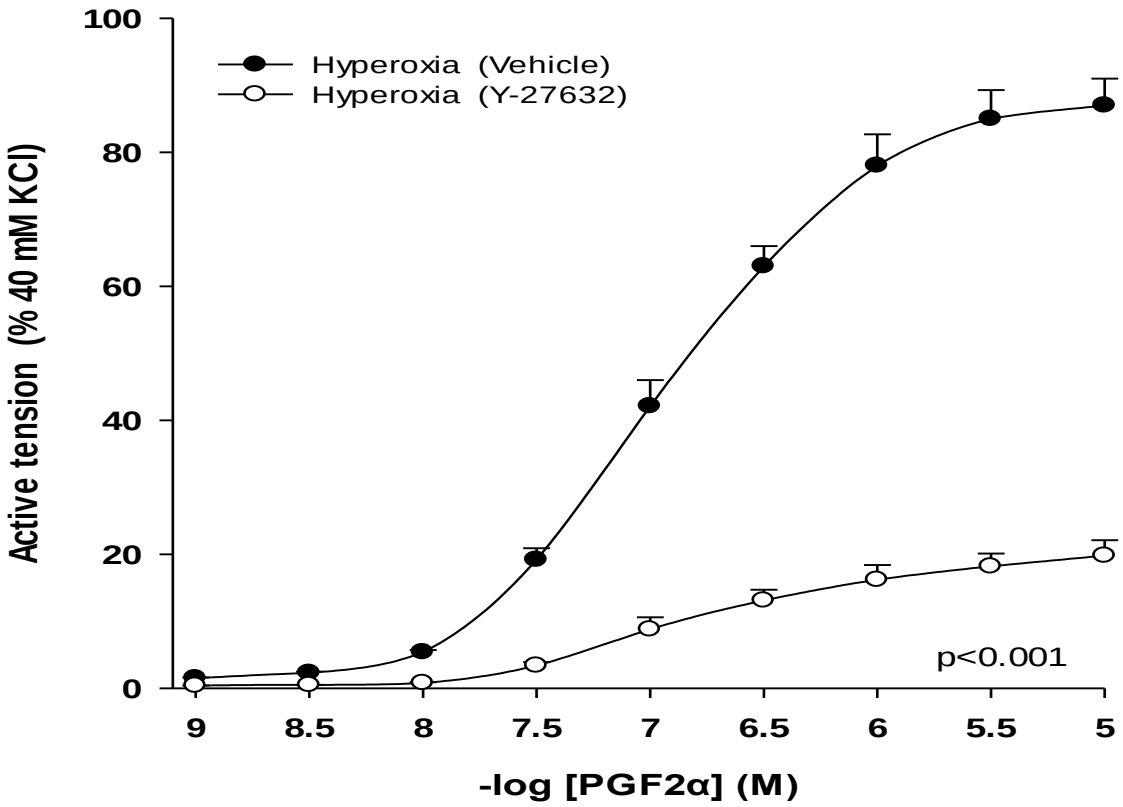

Figure 19. Effect of in vitro Rho-kinase inhibition by Y-27632 on* $PGF_2\alpha$ *-induced contraction of TSM of rat pups exposed to hyperoxia.

Use of Y-27632 (10 µM), significantly decreased the contractile responses of TSM toward $PGF_2\alpha$*. (Hyperoxia vs. Hyperoxia + Y-27632, P < 0.001). Data represent mean ± SEM of 10 experiments for each condition, and each performed in duplicate.*

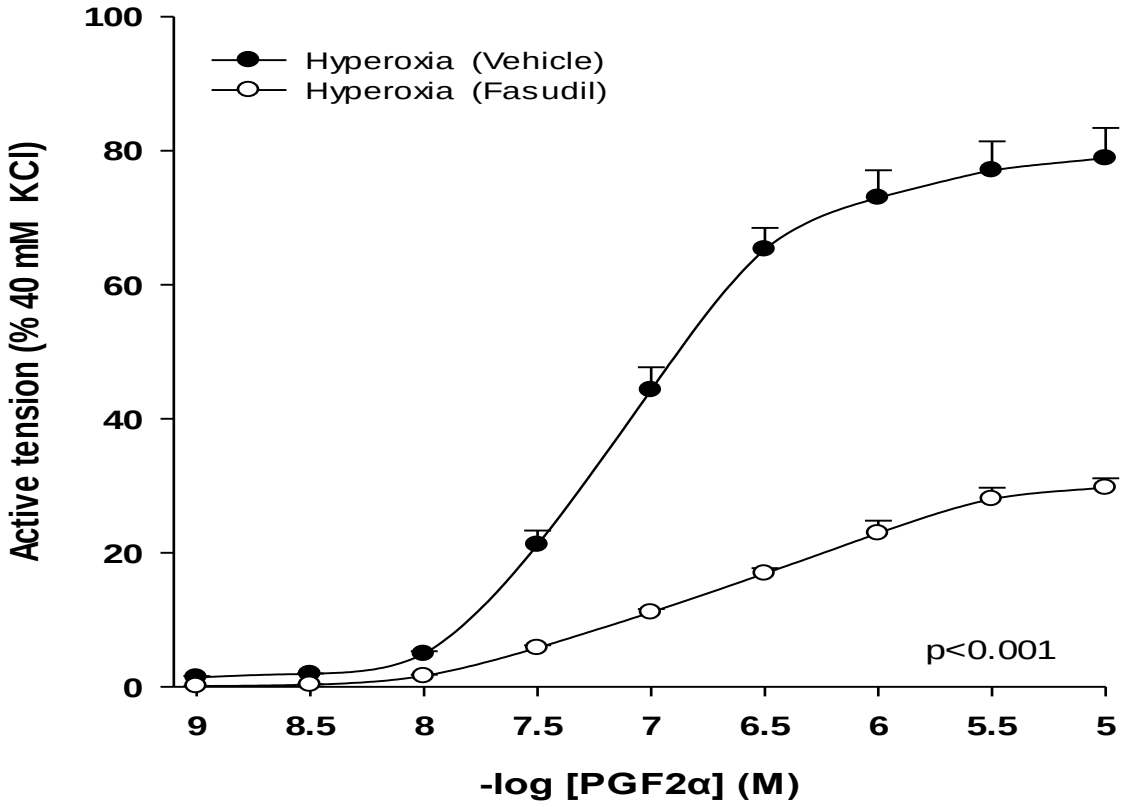

Figure 20. Effect of in vitro Rho-kinase inhibition by fasudil on* $PGF_2\alpha$ *-induced contraction of TSM of rat pups exposed to hyperoxia.

Use of fasudil (10 μM), significantly decreased the contractile responses of TSM toward $PGF_2\alpha$*. (Hyperoxia vs. Hyperoxia + Y-27632, P < 0.001). Data represent mean ± SEM of 10 experiments for each condition, and each performed in duplicate.*

6. DISCUSSION

To the best of our knowledge, this is the first study showing that targeting of Rho/Rho-kinase by using pharmacological inhibitors Y-27632 and fasudil attenuates hyperoxia-induced airway hyperreactivity and restores reduced relaxation in neonatal rats. In this study we demonstrated that Rho/Rho-kinase signaling plays an important role in adverse effects of hyperoxia.

BPD as a chronic lung disease that affects premature infants is characterized by interrupted lung development (84). There are number of characteristic features of BPD like alveolar simplification, dysmorphic lung vascularization (85, 86), lung inflammation, fibrosis (87), and as long-term effect of neonatal lung injury under this condition is increased airway reactivity in childhood and is most pronounced when there is a history of BPD (32, 33). Although, supplemental oxygen is often used as a life-saving therapy in preterm infants with respiratory failure; the excessive oxygen exposure or hyperoxia contributes to BPD pathogenesis. It is well established that exposure of rat pups to hyperoxia represents a good animal model of experimental BPD. Exposure of rat pups to hyperoxia was associated with pathophysiological features of BPD, including airway hyperreactivity (37-39, 47, 88). Also, the results of this study confirmed that hyperoxia increases ASM contractility towards a contractile agonist – methacholine. The etiology of airway hyperreactivity is multifactorial and there are many molecular players to contribute in this phenomenon. It was shown that hyperoxia upregulates arginase expression and activity (37, 39), an enzyme which competes for a common substrate with NOS, reducing bioavailability of substrate L-arginine to NOS, and subsequently decrease in NO production and disrupts NO/cGMP signaling (37, 39). This leads to decrease of relaxation (39) and presumably potentiating molecular key players involved in contraction of smooth muscle. Rho/Rho-kinase signaling as an important signaling pathway is involved in regulation of many processes within the cells such as cell proliferation, differentiation, chemotaxis, adhesion, invasion and smooth muscle contraction as well (89,90). Rho-kinase contributes to the increase of MLC_{20} phosphorylation via inhibition of MLCP and augmenting in this way the level of contraction via so called Ca^{2+}-sensitization (56, 57, 69-72).

Compelling evidence was accumulated supporting the implication of Rho/Rho-kinase signaling in the pathophysiological mechanisms of conditions characterized with airway hyperreactivity such as allergic asthma. Chiba et al. (75) have shown that Rho/Rho-kinase contributed functionally in contraction of ASM induced by acetylcholine, where the responses are increased in airways of rats challenged with allergen. A contribution of Rho-kinase in ASM

contraction was shown under in vitro preparations and in airway sensitivity under *in vivo* conditions in a guinea pig model of asthma (76).

In order to define the role of Rho/Rho-kinase signaling in hyperoxia-induced airway hyperreactivity in neonatal rats, the effect of two Rho-kinase inhibitors was tested under *in vitro* conditions. Both inhibitors, fasudil and Y-27632, in preparation of TSM reversed the increased contractile responses toward methacholine to the normal level, while did not affect contractile responses in tracheal preparations of normoxic animals. The ability of Rho-kinase inhibition to normalize increased contractile responses of TSM indicates that Rho/Rho-kinase signaling plays a crucial role on hyperoxia-induced airway hyperreactivity and further its implication on BPD development. Involvement of Rho/Rho-kinase signaling on airway hyperreactivity was showed in tracheal smooth muscle of guinea pigs sensitized in a passive manner (91). In this study Rho-kinase inhibitor Y-27632 normalized increased contractile responses toward histamine and methacholine (91).

In addition to the effect of Rho-kinase inhibition on airway hyperreactivity induced by hyperoxia tested under the *ex vivo* conditions, we tested the *in viv*o effect of Rho-kinase inhibition by supplementing animals with Y-27632 or fasudil (10 mg/kg/day) parallel to hyperoxic exposure. Supplementation of animals with Rho-kinase inhibitors prevented airway hyperreactivity in hyperoxic animals. Rho-kinase inhibition did not have an effect on the contractile responses in room air animals, and our explanation is that this might be because the involvement of Rho/Rho-kinase signaling in regulation of smooth muscle contraction is more potentiated in pathophysiological conditions rather than under normal conditions where the contraction is primary controlled by Ca^{2+}-dependent phosphorylation of MLC. This might be also connected to the fact that role of Rho/Rho-kinase signaling in contraction is agonist dependent and receptor-dependent (76, 92) which might change under different conditions.

Our data agree with previous studies that targeted Rho-kinase. The protective effect of Rho-kinase inhibitor was showed in actively sensitized mice, where Y-27632 provided *in vivo* suppressed the increased airway responsiveness induced by ovalbumin and/or syncytial virus (78). A reversal effect of Y-27632 was shown against allergen-induced airway hyperresponsiveness after asthmatic reactions in guinea pigs (77). Rho-kinase inhibition was showed to have a protective effect against pulmonary hypertension induced by neonatal hyperoxia (93). It was proved that hyperoxia increases protein expression of Rho-kinase and its activity as well as myosin-binding subunit of myosin-associated phosphatase type-1 (MYPT1)

in lung tissue of rats, while treatment of animals with Rho-kinase inhibitors Y-27632 or fasudil prevented these effects of hyperoxia (93, 94).

To further continue with determining the role of Rho/Rho-kinase signaling in the mechanics of ASM under hyperoxic conditions we investigated the effect of hyperoxia in relaxation of TSM, in absence or presence of Rho-kinase inhibitors supplemented *ex vivo* to isolated tracheal preparations or *in vivo* to the animals. Our data show that hyperoxia impairs EFS-induced relaxant responses of TSM as compared to control responses obtained from room air animals. This is in agreement with previous studies confirmed that neonatal hyperoxia decreased relaxation of intrapulmonary (39) and extra-pulmonary (36) ASM. Inhibition of Rho-kinase using either Y-27632 or fasudil restored impaired relaxant responses in hyperoxia-exposed animals to normal level. Hyperoxia reduces the substrate bioavailability to NOS, hence NO deficiency, which disturbs NO/cGMP signaling and decreases smooth muscle signaling (39). Hyperoxia decreases also the cAMP level (38), impairing thus relaxant responses and enhancing contraction of ASM in lung parenchymal strips of rat pups. This is because inhibition of cAMP/PKA signaling pathway, as it is known that cAMP activates PKA, which in turn phosphorylates membrane and/or intracellular proteins involved in process of promotion of ASM relaxation and bronchodilation of contracted airways (95, 96).

cAMP/PKA signaling induces Ca^{2+}-desensitization via activation of MLCP-associated system (97). While it is known that hyperoxia decreases level of cAMP, consequently it upregulates Ca^{2+}-sensitization and hence augments the role of Rho/Rho-kinase signaling in smooth muscle contraction in experimental model of BPD which is associated with increase of contraction and decrease of relaxation of ASM. Therefore, the right mechanism to reverse the effects of hyperoxia on ASM is minimization Rho/Rho-kinase signaling. Rho-kinase inhibitors Y-27632 and fasudil in this study applied either *ex vivo* or *in vivo* showed to be effective to reverse the effects of hyperoxia. Y-27632 was shown itself to induce relaxation of preconstricted rabbit TSM and human bronchial smooth muscle (98).

Growth factors are known for contributing in airway remodeling and lung fibrosis in different diseases including BPD (99, 100). There is the evidence indicating that growth factors via their receptors can activate Rho/Rho-kinase pathway directly, triggering in that way to contraction of smooth muscle (56, 63). It was shown that EGF induces contraction of TSM of guinea pigs via arachidonic acid metabolism involving tyrosine kinase receptors and phospholipase A2 (101). EGF binds to its receptors with intrinsic tyrosine kinase activity and activates MEK and

MAPK, resulting in increase of arachidonic acid (AA) via cytosolic phospholipase A_2 ($cPLA_2$) activation. Through COX, AA will be converted to prostaglandins, such as PGE_2 and $PGF_2\alpha$. These prostaglandins in turn may bind to their receptors and induce ASM contraction which is Rho kinase-dependent (102). In this study we investigated the effect of hyperoxia on EGF-induced contraction of ASM and in this line we tested the effect of Rho-kinase, COX and MEK inhibitors on EGF-induced contraction. Using different concentrations of EGF, we confirmed that EGF induces TSM contraction in rat pups TSM which were higher in hyperoxia-exposed animals than room air-exposed animals. Inhibition of these enzymes almost eliminated the contractile responses of ASM toward EGF. These results indicate that contractile responses of EGF on rat pup TSM are Rho/Rho-kinase-dependent signaling and it exerts the effect via contractile prostaglandins. Therefore, next logical step was to investigate the effect of $PGF_2\alpha$ on ASM contraction under hyperoxic conditions and the effect of Rho-kinase inhibitor in these responses. Our results show that hyperoxia increased contractile responses of rat pup TSM toward $PGF_2\alpha$, and these responses were decreased but not completely blocked by Rho-kinase inhibitors (Y-27632 and fasudil). Our results are in accordance with previous findings of other authors, who showed that growth factors, such as insulin-like growth factor-1 (IGF-1), EGF, platelet-derived growth factor (PDGF) induced contraction responses in human bronchial smooth muscle and guinea pig TSM (102, 103). Growth factors have been found to be implicated in the inflammation of airways and lung tissues and are synthetized and released by inflammatory cells, and the expression level of EGF and its receptors have been found elevated in asthmatic airways (104). Based on the fact that growth factors are involved in repairing processes and are released by inflammatory cells, increased levels of growth factors under conditions associated with lung and airway inflammation such as BPD growth factors may in turn potentiate the airway hyperreactivity mediated by Rho/Rho-kinase signaling. Other studies have also shown the contractile effect of $PGF_2\alpha$ on ASM of guinea pigs and PDGF induced $PGF_2\alpha$ production from tracheal smooth muscle preparations which was decreased by inhibition of COX or MEK (103).

The histopathological changes in lung tissue of rat pups exposed to hyperoxia such as alveolar collapse, obvious alveolar wall thickening, and collagen deposition were prevented by fasudil treatment of rat pups exposed to hyperoxia (94). Fasudil has been used also in clinical trials to treat hypertension where it was shown to be effective to treat this medical condition (105), and coronary artery spasm (106).

The limitations of this study are that we could not complement the physiological and pharmacological studies with molecular part to follow the protein as well as mRNA expression of this molecular players implicated in airway hyperreactivity.

Taken altogether from results of this study we can confirm that Rho/Rho-kinase signaling pathway has an important role on hyperreactivity induced by neonatal hyperoxia thus contributing to BPD development, therefore we speculate that targeting Rho-kinase by using Rho-kinase inhibitors may provide an effective therapy parallel with hyperoxia treatment, which could be beneficial for premature babies.

7. CONCLUSION

The results of this study conclude as follow:

1. Overall, from results we conclude that Rho/Rho-kinase signaling pathway is involved in airway hyperreactivity induced by hyperoxic exposure.

2. Hyperoxia enhances contractile responses of tracheal smooth muscle when compared to room air exposed animals.

3. Hyperoxia impairs relaxation of tracheal smooth muscle compared with room air animals.

4. Supplementation of rat pups or tracheal preparations with Rho-kinase inhibitors *in vitro* or *in vivo* protects against hyperoxia-induced enhancement in airway contractile responses.

5. Supplementation of rat pups or tracheal preparations with Rho-kinase inhibitors *in vitro* or *in vivo* restores hyperoxia-induced decrease in airway relaxant responses.

6. Hyperoxia potentiates growth factor-induced contractile responses of airway smooth muscle via Rho/Rho-kinase signaling pathway.

7. Hyperoxia potentiates PGF2α-induced responses of airway smooth muscle via Rho/Rho-kinase signaling pathway.

8. Based on the results we speculate than use of Rho-kinase inhibitors may serve as an effective therapy to counteract the adverse effects of neonatal hyperoxia in preterm babies.

8. SAŽETAK (ABSTRACT IN CROATIAN)

***Uvod*:** Bronhopulmonalna displazija (BPD) je kronična bolest pluća koja se pojavljuje u prematurno rođene djece. Stanje je karakterizirano hiperreaktivnošću dišnih puteva i plućnim oštećenjima. Pokazano je da je Rho/Rho-kinazni signalni put uključen u bolestima plućnih puteva.

Ciljevi: Cilj istraživanja je odrediti ulogu Rho/Rho-kinaze u patofiziologiji BPD u experimentalnom štakorskom modelu te učinke farmakoloških inhibitora ovoga puta.

Materijali i postupci: Četvrtog dana štakorski mladunci su izloženi hiperoksiji (>95% kisika), odnosno sobnom zraku, kroz 7 dana. Nekim skupininama životinja su za vrijeme izlaganja tim uvjetima intraperitonejski uštrcavani inhibitori Rho-kinaze, Y-27632, odnosno, fasudil. 12-tog dana životinje su eutanazirane i trahealni cilindri su priređeni za in vitro mjerenja sila kontrakcije glatke muskulature traheje (GMT), potaknute različitim dozama metakolina (MK), zatim epitelnog čimbenika rasta, prostaglanina F2α; u uvjetima sa i bez inhibitora Y-27632 (10mM), odnosno, fasudil (10mM). Za testirane odgovora relaksacije, uzorci su bili prekontrahirani s pomoću betanekola, te potom stimulirani električnim poljem u uvjetima sa i bez inhibitora.

Rezultati: U hiperoksičnim skupinama životinja kontrakcijski odgovor na MK je bio značajno povećan ($p<0,001$) u usporedbi s kontrolnom skupinom. Maksimalni kontrakcijski odgovor je bio 1,8 +/- 0.17 g u hiperoksičnih, a 1,3 +/- 0.15 g u kontrolnoj skupini. Primjena, in vivo i in vitro, inhibitora Rho-kinaze je zakočila učinke hiperoksije i vratila reaktivnost na normalnu razinu. Relaksacijski GMT-odgovori su značajno niži ($p<0,001$) u hiperoksičkoj skupini u odnosu na kontrolu, a maksimalna vrijednost (na 20V) je bila 38,8 +/- 4,2%, odnosno 82,02 +/-6,2 %. Primjena inhibitora je dovela do ponovne uspostave relaksirajućeg odgovora, i u in vivo i u in vitro uvjetima. Hiperoksija pojačava GMT-kontraktilnost induciranu s pomoću EGF i PGF2 α, a inhibitori Rho-kinaze normaliziraju kontraktilni odgovor pokrenut tim agonistima.

Zaključak: Ova istraživanja upućuju da Rho/Rho-kinazni signalni put ima važnu ulogu u hiperoksijom potaknutoj hiperreaktivnosti dišnih putova. Farmakološke intervencije na razini ovoga signalnoga puta mogu biti osnova za potencijalnu kliničku primjenu inhibitora kod prematurne djece liječene hiperoksijom.

Ključne riječi: glatki mišići dišnih putova, bronhopulmonalna displazija, kontrakcija mišića, EFS, EGF, fasudil, relaksacija mišića, Rho-kinaze, traheaani glatki mišić, Y-27632.

9. ABSTRACT

Role of Rho/Rho-kinase signaling pathways in development of bronchopulmonary dysplasia in the experimental rat model, Qëndresa Beqiraj–Zeqiraj, 2022

***Introduction*:** Bronchopulmonary dysplasia (BPD) is a chronic lung disorder that is most common among children born prematurely, is characterized with airway hyperreactivity and lung injury. Rho/Rho-kinase signaling recently has received an increased attention in airway diseases.

Aims: The aim of the study was to determine the role of Rho/Rho-kinase in pathophysiology of experimental BPD and the effect of pharmacologically targeting of this pathway.

***Materials and methods*:** At the 4th day of life rat pups were exposed either to hyperoxia (>95% O_2) or room air for 7 days. Some sets of animals during exposure received i.p. Rho-kinase inhibitors *Y-27632* or *fasudil*. At 12th day of life animals were euthanized and tracheal cylinders were prepared for *in vitro* force measurement to study contraction of tracheal smooth muscle (TSM) toward different doses of methacholine (MCh); epithelial growth factor or prostaglandin F2α in absence or presence of *Y-27632 (10 μM)* or *fasudil (10 μM)*. For relaxant responses preparations were pre-contracted with bethanechol then treated with electrical field stimulation (EFS) in absence or presence of *Y-27632* or *fasudil*.

***Results*:** In hyperoxia-exposed animals the contractile responses of TSM toward MCh were significantly increased (*P<0.01*) compared to room air-exposed animals, and the maximal values of contractile responses were 1.8 ± 0.17 g in hyperoxia group and 1.13 ± 0.15 g, respectively. Treatment with Rho-kinase inhibitors either *in vitro* or *in vivo* reversed this effect of hyperoxia and normalized responses to control level. Relaxant responses of TSM were significantly decreased (*P<0.001*) in hyperoxic group of animals compared with room air group, and maximal values of relaxant responses (at 20V) were 38.80 ± 4.20% and 82.02 ± 6.20 % at 20V, respectively. Treatment with Rho-kinase inhibitors either *in vitro* or *in vivo* restored the impaired relaxation in hyperoxic group to normal level. Hyperoxia potentiated the contractile effect of exogenous EGF and PGF2α on TSM, while presence of Rho-kinase inhibitors reversed the contractile responses induced by these agonists.

***Conclusion*:** This study prove that Rho/Rho-kinase signaling plays an important role in airway hyperreactivity induced by hyperoxia, and pharmacological targeting of this pathway provides an effective therapeutic approach to prevent the adverse effects of neonatal hyperoxia.

Key words: airway smooth muscle, bronchopulmonary dysplasia, contraction, EFS, epidermal growth factor, fasudil, relaxation, Rho-kinase, tracheal smooth muscle, Y-27632.

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11. CURRICULUM VITAE

Qëndresa Beqiraj–Zeqiraj was born on December 12, 1983, in Prishtina, Kosovo, where she finished primary school and gymnasium.

In 2008, she graduated from the Faculty of Medicine, University of Prishtina. During bachelor studies, was awarded from the University of Prishtina with high grades average.

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Co- author of 2 papers published in peer reviewed international journals.

Scientific associate at National Research Project: PGE2-EP Signaling Pathway Hyperoxia-Induced Impaired Airway Relaxation financed by Ministry of Education and Science - Republic of Kosovo; 2014-2015.

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