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RESEARCH ARTICLE

Hypoxia Promotes Proliferation and Inhibits Apoptosis of Pulmonary Arterial Smooth Muscle Cells via Modulating TRPC6

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A B S T R A C T

Ascites syndrome (AS) is a common nutritional metabolic disease in broilers that results in major loss to the breeding industry. The occurrence of AS is closely related to the abnormal proliferation of pulmonary artery smooth muscles cells (PASMCs) caused by hypoxia. The transient receptor potential cation channel subfamily C member 6 (TRPC6) is a Ca^{2+} channel situated on cell membranes, and belongs to the proliferation of PASMCs caused by hypoxia in mammals. However, its role in hypoxic PASMCs in broilers remains unclear. Here, we investigated the effects of TRPC6 on the proliferation and apoptosis of primary chicken PASMCs under hypoxic conditions using an *in vitro* model. Primary chicken PASMCs were cultured under normoxic or hypoxic conditions (3% O_2). The hypoxic cells were treated with 10 µM of SKF 96365 or 50 µM of fluofenamic acid (FFA) to inhibit or activate TRPC6, respectively. Cell viability was detected by CCK-8, intracellular Ca^{2+} levels, cell cycle and cell apoptosis were assayed by a flow cytometric assay, the mRNA levels of TRPC6 were measured by digital-droplet PCR (ddPCR), the protein levels of TRPC6 were tested by immunoblotting, and the mRNA levels of caspase3 were detected by RT-PCR. Our results revealed that exposing PASMCs to hypoxic conditions for 48h increased cell viability and intracellular Ca^{2+} levels. Additionally, hypoxic conditions increased the expression of TRPC6 and promoted cell cycle progression, but decreased cell apoptosis and caspase 3 mRNA levels. When the hypoxic PASMCs were treated with SKF 96365, inhibition of TRPC6 and cell proliferation and promotion of apoptosis were observed only in the first 24h of treatment. Treatment with FFA for 24h had the opposite effects. These results suggested that TRPC6-mediated Ca^{2+} entry contributed to hypoxia-induced PASMCs proliferation and apoptosis resistance, which identified TRPC6 as a possible key target in vascular remodeling in chicken.

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INTRODUCTION

Ascites syndrome (AS) is also known as pulmonary hypertension, which is a nutrition metabolic disease and commonly found in fast-growing broilers (Kamely *et al*., 2015). Low oxygen concentrations in the environment and increase in tissue demands for oxygen increase the risk of AS in broilers (Kamely *et al*., 2015). In humans, hypoxia is one of the main causes of pulmonary hypertension (Sylvester *et al*., 2012). Several studies suggest that chronic hypoxia or areas of high altitudes result in pulmonary hypertension (Zhang *et al*., 2018; Riley and Sciurba, 2019). Pulmonary arterial remodeling is a pathological characteristic of pulmonary hypertension (Tan *et al*., 2018). Vascular remodeling is mainly manifested by abnormal proliferation of the inner, middle and outer layers of pulmonary artery (Voelkel and Tuder, 2000). PASMCs, as the main component of the middle

layer of the pulmonary artery, play a key role in pulmonary vascular remodeling. Excessive proliferation of PASMCs was observed during the development of pulmonary hypertension and was identified as the main cause of increased pulmonary vascular resistance caused by hypoxia (Guignabert and Dorfmuller, 2013). Furthermore, vascular remodeling is often studied using *in vitro* models by culturing PASMCs under hypoxic conditions (Zhang *et al*., 2019). Using *in vitro* models of mammals, increased proliferation and inhibition of apoptosis were observed under hypoxic conditions (Gui *et al*., 2017). However, there are few studies analyzing the effects of hypoxia on the proliferation and apoptosis of PASMCs in broilers *in vitro*.

Intracellular Ca^{2+} is an important second messenger in cells involved in intracellular signal transduction, cell proliferation and apoptosis. Numerous studies demonstrated that a rise in intracellular Ca^{2+} levels triggers the proliferation of mammalian PASMCs under hypoxic conditions (Kamely *et al*., 2015). TRPC, an important calcium channel on cell membrane, is considered to be involved in the regulation of calcium concentration in PASMCs. TRPC6 is an important subtype of TRPC family expressed in the PASMCs and is closely related to the occurrence of pulmonary hypertension. Enhanced activity of the TRPC6 channel is involved in PASMC proliferation induced by hypoxia and knockout of TRPC6 showed beneficial phenotypes in rodent models of pulmonary hypertension (Xia *et al*., 2014). However, it is not certain whether TRPC6 has the same function in chicken PASMCs under hypoxia.

Thus, this study was designed to determine whether hypoxia has a stimulatory effect on chicken PASMC proliferation through TRPC6-mediating elevated intracellular Ca^{2+} levels using an *in vitro* model.

MATERIALS AND METHODS

Cell culture: The study was approved by Animal Ethics Care Committee of South China Agriculture University. PASMCs were isolated and cultured based on previous studies (Yang *et al*., 2013). Healthy broiler chickens (7-10 days of age) were euthanized by cervical dislocation, and main pulmonary arteries (PA) were isolated and washed in Hanks balanced salt solution. Next, the PA was cut into 1 mm^3 and digested using collagenase type II (1 mg/mL) at 37°C for 100 mins before collecting cells. Cells were washed twice and seeded in DMEM containing 10% fetal bovine serum (FBS) (Pan, Bagolia, Germany) in a 95% air and 5% CO_2 at 37°C incubators.

Treatment and proliferation: Cells were seeded and allowed to grow until reaching confluence. Cells were cultured under normoxic (95% ambient air; 5% CO_2) or hypoxia environments (5% CO_2, 3% O_2 and 92% N2). Then, hypoxic cells were treated with 10 µM of SKF 96365 or 50 µM of fluofenamic acid (FFA).

The cells seeded in 96-well plates and 10 µL of CCK-8 was added into each well to incubate it for 1 hour. Whereas, absorbance in each well was investigated by microplate reader (Bio-Tek, Vermont, USA).

Cell cycle analysis: Cells with indicated treatments were harvested and resuspended in 70% ethanol at 4°C overnight. Then, cells were washed with PBS and stained with 1 µL of RNaseA (10 mg/ml), 15 µL of 25×propidium iodide (CoWin Biosciences, Beijing, China) and 400 µL of staining buffer (CoWin Biosciences, Beijing, China) in the dark at RT for 30 min. Finally, cells were analyzed using a flow cytometer (Beckman Coulter, California, USA).

Apoptosis: Cells were harvested in 100 µL of staining buffer solution, while 5 µL of Annexin-V-FITC and 5 µL of propidium iodide were added, which was then incubated for 15 min at 37°C. Then, 400 µL of staining buffer solution was added to the cell suspension. Cells were analyzed at 525 and 620 nm to quantify apoptosis.

Droplet digital PCR (dd-PCR) and RT-qPCR: Total RNA was taken out by using Trizol reagent from the cells. RNA was reverse transcribed to complementary DNA using a Prime Script reverse transcriptase reagent kit (TaKaRa, Dalian, China). The TRPC6 primer included: forward: CTCGGTCAAAGGAAGCAATC, and reverse: CACCTTCATTGACTTCGTCAC. The GAPDH primer included forward: CTGTGACTTCAATGGTGACAGC, and reverse: CTTGGATGCCATGTGGACC. The mixture was combined with BioRad droplet oil to generate nano droplets, then transferred to a 96-well PCR plate and ddPCR reactions were performed. Fluorescent signal of the droplets was analyzed using Quantasoft™ version 1.7.4 software (Bio-Rad, California, USA).

For RT-qPCR, cDNA was amplified using a ChamQ SYBR qPCR Master Mix Kit (Vazyme, Nanjing, China) and a Real-time System (Roche 480, Basal, Switzerland). Amplification was performed using 3-step cycling conditions with specific primer sequence for caspase 3: forward: CGGACTGTCATCTCGTTCA and reverse: TGGCTTAGCAACACACAAAC.

Western blotting: Western blotting was performed according to previous methods (Liao *et al*., 2019). GAPDH was used as an endogenous loading control. Primary antibodies (Boster Biological Technology, Wuhan, Hubei, China) recognizing GAPDH and TRPC6 were used. Blots were visualized using chemiluminescence western blotting substrate kit (Beyotime Biotechnology, Songjiang, Shanghai, China).

Statistical analysis: Results were articulated as mean ±SD. Data were investigated using one-way ANOVA. All data analyses were performed using SPSS version 24.0 for Windows (SPSS Inc., Chicago, IL, USA). The 'Wu Kong' platform (https://www.omicsolution.org/wkomics/main/) was used for relative Pearson correlation analysis. Changes were stated to be statistically significant when $P<0.05$.

RESULTS

Expression of TRPC6 and intracellular Ca^{2+} levels in hypoxic PASMCs: First, we measured TRPC6 mRNA and protein levels as well as intracellular Ca^{2+} levels to observe the effects of hypoxia on the physiological state

of the PASMCs. As shown in Fig. 1A to E, hypoxia increased TRPC6 mRNA, TRPC6 protein and intercellular Ca^{2+} levels at 48h compared to the control group (P<0.01). However, intercellular Ca^{2+} levels and TRPC6 expression levels were not significantly changed when cells were exposed to an anoxic environment for 24 h (P>0.05).

Viability, cell-cycle and apoptosis in hypoxic PASMCs: As shown in Fig. 2A, cell viability was not altered in the hypoxia group compared to the control group at 24 h (P>0.05). However, when the duration of hypoxia was prolonged to 48 h, cell viability was significantly increased compared to the control group (P<0.01) (Fig. 2A). Moreover, cells in the S phase notably increased (P<0.01) while cells in G0/G1 phase decreased in the hypoxia group compared to the control group after exposed to hypoxia for 48 h (P<0.05) (Fig. 2B and C). However, the number of PASMCs in every cell cycle phase did not noticeably change between the hypoxia and control groups (P>0.05) (Fig. 2B and C). The apoptotic ratio of PASMCs was measured using the Annexin-V-FITC/PI double staining. After 48 h of hypoxia initiation, the proportion of early apoptotic PASMCs and all apoptotic PASMCs decreased in the test group (P<0.01) (Fig. 2D and E). Nevertheless, no differences in apoptotic rates were observed between the control and the hypoxia group after 24h of hypoxia incubation. (Fig. 2D and E). Finally, RT-PCR results revealed that caspase3 mRNA levels were markedly lower in the test group compared to the control group (P<0.01) (Fig. 2F). These results revealed that hypoxic PASMCs show accelerated cell cycle progression and reduced apoptosis.

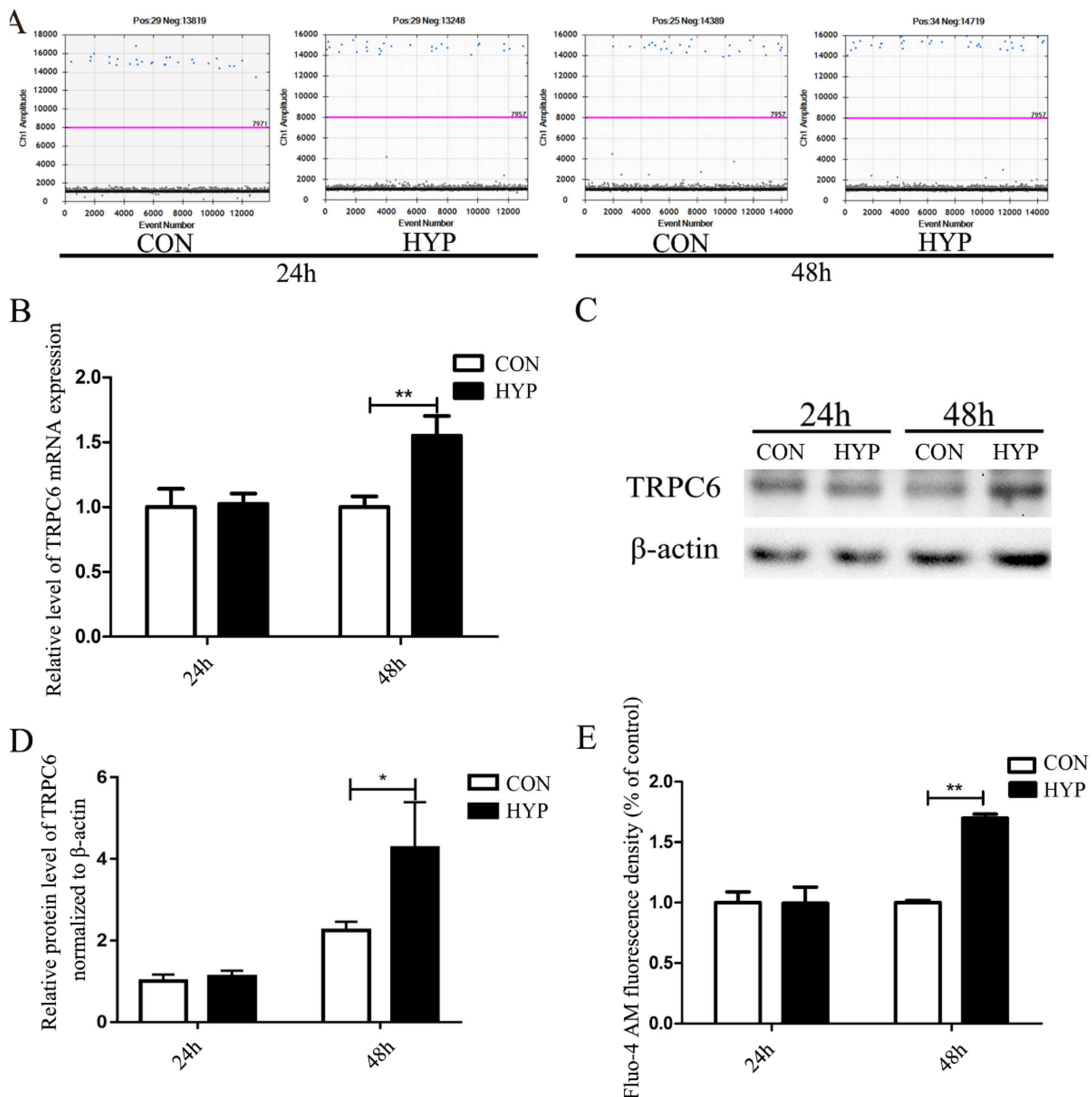

Fig. 1: Intracellular Ca^{2+} levels and mRNA level of TRPC6 in PASMCs. (A) dd-PCR output showing mRNA levels of TRPC6. Dots represent emulsion droplets. Blue points considered as positive signals while Black points as negative signals by the system. (B) Quantification of dd-PCR results. (C) TRPC6 protein expression. (D) Densitometric analysis of TRPC6 protein expression levels. (E) Measures the intracellular Ca^{2+} level. The data are presented as the mean ± SD, n=3, * P<0.05, ** P<0.01. CON, control group; HYP, cells cultured under hypoxic conditions.

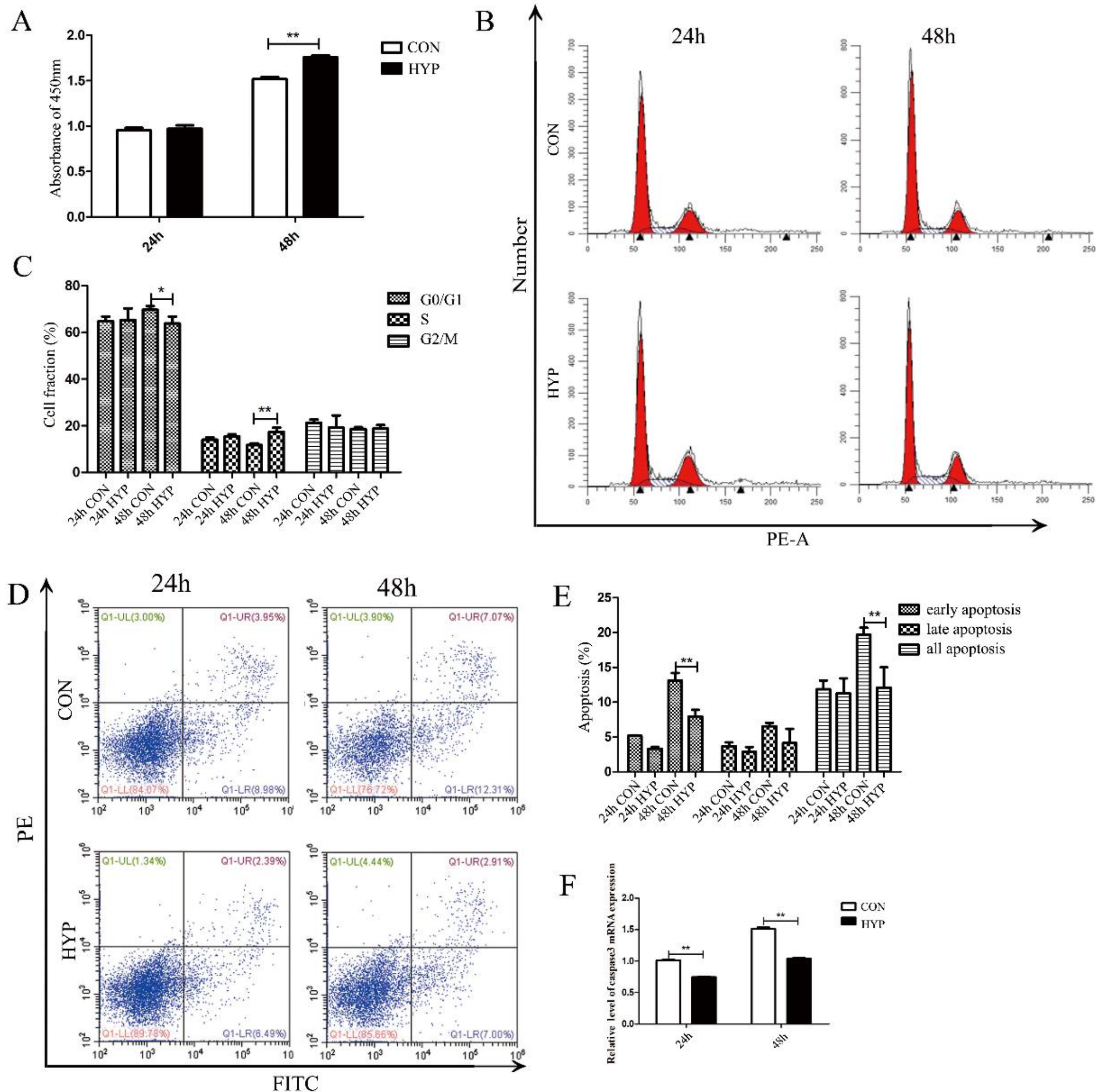

Fig. 2: Effect of hypoxia on cell viability, cell cycle and apoptosis. (A) Cell viability after treatment with hypoxia. (B) The cell cycle was analyzed using flow cytometry after PASMCs were cultured for 24 h and 48 h under normoxia and hypoxia. (C) Cell cycle distribution presented as % at different cell cycle stages evaluated by flow cytometry. (D) Flow cytometric analysis of apoptosis. (E) Comparison of apoptosis between normoxia control group and hypoxia groups at 24 and 48 h. (F) RT-PCR analysis the expression of caspase3 mRNA in PASMCs. The data are presented as the mean ± SD, n=3, * P<0.05, ** P<0.01. CON, control group; HYP, cells cultured under hypoxic conditions.

Effects of TRPC6 intracellular Ca^{2+} levels: After PASMCs were treated with SKF 96365 (10 μM) or FFA (50 μM) at 24h and 48h under hypoxic conditions, we analyzed TRPC6 mRNA and protein levels. As shown in Fig. 3A and B, treatment with SKF 96365 (10 μM) reduced hypoxia-induced TRPC6 mRNA expression in PASMCs at 24h and 48h under hypoxic conditions (P<0.05). TRPC6 mRNA levels were significantly increased after FFA (50 μM) treatment at 48h compared to the HYP group (P<0.05). Furthermore, TRPC6 protein levels decreased after SKF 96365 treatment for 24h (P<0.01). Then, we investigated intracellular Ca^{2+} levels in PASMCs. As shown in Fig. 3E, intracellular Ca^{2+} levels were increased in the FFA group (P<0.05), but a significant decrease in intracellular Ca^{2+} levels were observed in the SKF group (P<0.01) when compared to the HYP group after 24h of hypoxia (Fig. 3A). Intracellular Ca^{2+} levels showed no differences among the HYP and SKF groups, as well as among the HYP and FFA groups after 48h of hypoxia (P>0.05) (Fig. 3A). Thus, these data revealed that TRPC6 expression was positively correlated with intracellular Ca^{2+} concentrations.

Effects of TRPC6 on cell proliferation and apoptosis in hypoxic PASMCs: As shown in Fig. 4A, repressed expression of TRPC6 decreased cell viability (P<0.01). Cell viability was effectively inhibited in PASMCs treated with FFA compared to the HYP group under hypoxic conditions at 24h (P<0.01). Cell viability did not change in the FFA group compared with the HYP group when cells were exposed to hypoxia at 48h (P>0.05). We also found that cells in the G0/G1 phase decreased significantly

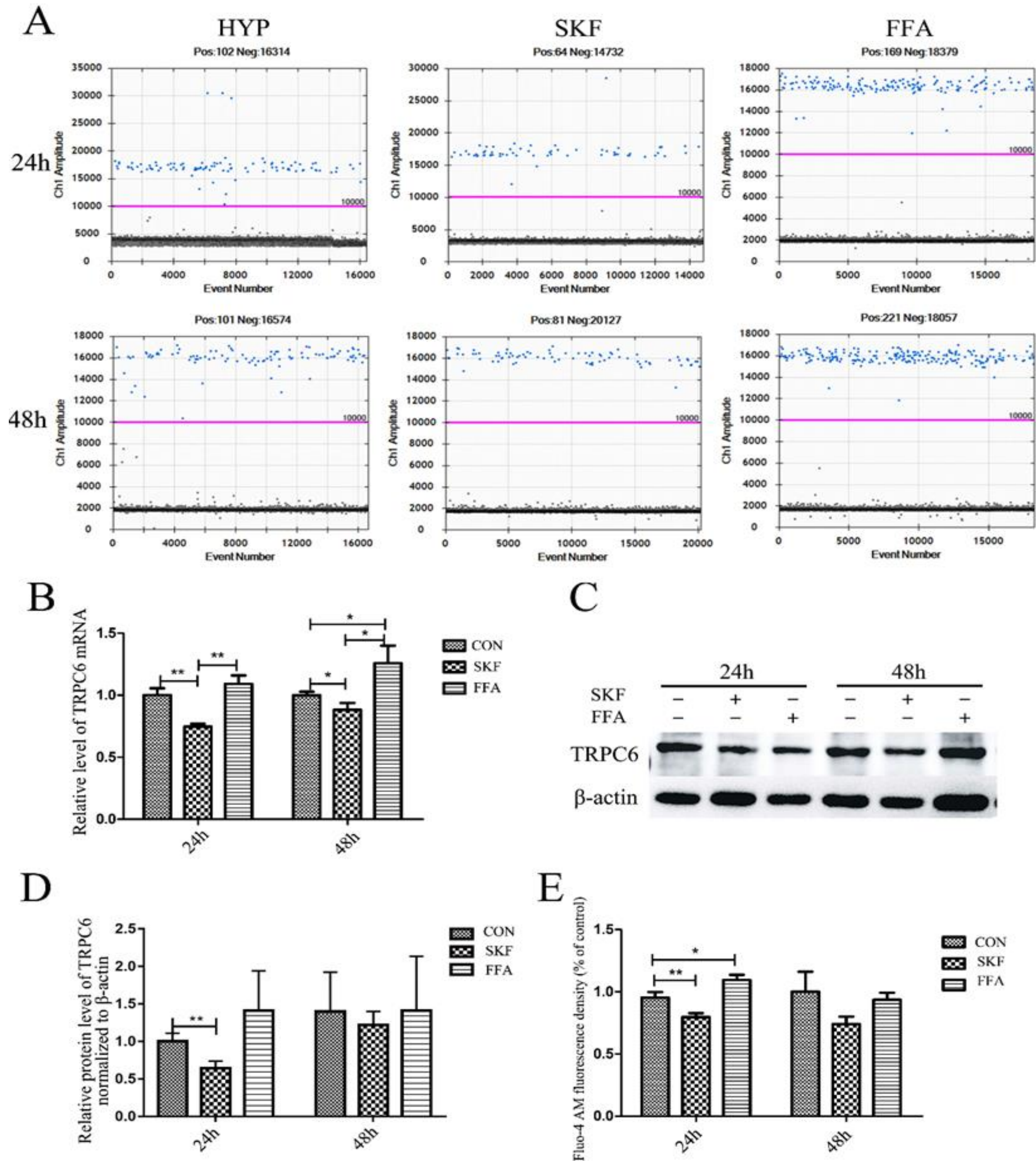

Fig. 3. Effects of TRPC6 activators or inhibitors on the expression of TRPC6 and intracellular calcium levels. Cells were treated with SKF 96365 or FFA for 24 h and 48 h under hypoxia. (A) dd-PCR output showing mRNA levels of TRPC6. Dots represent emulsion droplets. Blue points considered as positive signals while Black points as negative signals by the system. (B) Quantification of dd-PCR results. (C) TRPC6 protein expression. (D) Densitometric analysis of TRPC6 protein expression levels. (E) Measures the intracellular Ca^{2+} level. The data are presented as the mean ± SD, n=3, * P<0.05, ** P<0.01. HYP, cells cultured under hypoxic conditions; SKF, cells treated with 10 μM SKF 96365 under hypoxia; FFA, cells treated with 50 μM FFA under hypoxia.

in the SKF group compared to HYP group (P<0.01), but the rate of cells in the G2/M phase increased significantly in the SKF group compared with the HYP group under hypoxic conditions at 24h (P<0.01) (Fig. 4B and C). Furthermore, FFA significantly increased the proportion of cells in the S phase compared with the HYP group under hypoxia conditions at 24h (P<0.05) (Fig. 4B and C). However, cell cycle analysis did not show appreciable differences in the HYP, SKF and FFA groups after 48h of hypoxia (P>0.05) (Fig. 4B and C). Furthermore, apoptosis rates were examined using flow cytometry. As shown in Fig. 4D and E, there was a decrease in the ratio of early apoptotic cells and all apoptotic cells when PASMCs were treated with FFA under hypoxic conditions for 24h (P<0.01). However, when cells were treated with FFA under hypoxic conditions for 48h, the number of apoptotic cells did not differ when compared to the HYP group (P>0.05; Fig. 4E). In addition, treatment with SKF 96365 increased the number of apoptotic cells but there were no significant differences observed (P>0.05; Fig. 4E). As shown in Fig. 5F, expression levels of caspase3 mRNA were significantly lower when treated with SKF

Fig. 4: Effect of TRPC6 activators and inhibitors on cell cycle and apoptosis of hypoxic PASMCs. (A) Cell viability was monitored with CCK-8 assay at the indicated time after treatment with SKF 96365 or FFA in PASMCs under hypoxia. (B) Cytometric analysis of cell cycle distribution. (C) Statistical analysis showing the changes in cell cycle progression. (D) Flow cytometric analysis of apoptosis. (E) Percentage of early apoptosis cells, later apoptosis cells and all apoptosis cells in PASMCs. (F) The expression of caspase3 mRNA in PASMCs. The data are presented as the mean ± SD, n=3, * P<0.05, ** P<0.01. HYP, cells cultured under hypoxic conditions. SKF, cells treated with 10 μM SKF 96365 under hypoxia. FFA, cells treated with 50 μM FFA under hypoxia.

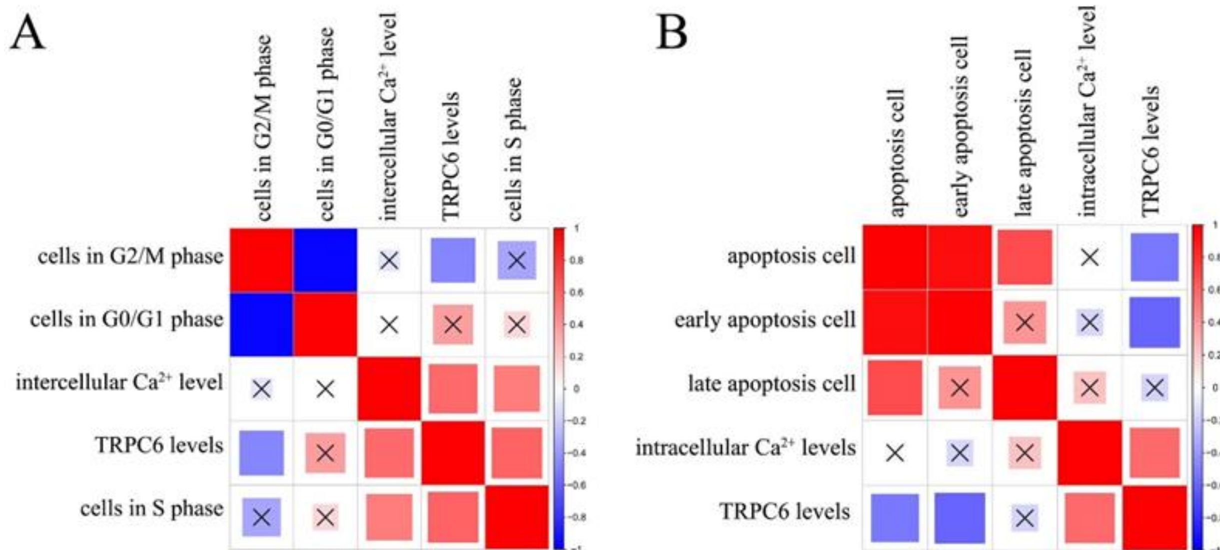

Fig. 5: Intracellular Ca^{2+} levels, TRPC6 mRNA levels, cell proliferation and cell apoptosis were mutually correlated in PASMCs and calculated using Pearson's correlation tests. (A) Correlation analysis among intercellular Ca^{2+} levels, TRPC6 levels and cell proliferation. (B) Correlation analysis among intercellular Ca^{2+} levels, TRPC6 levels and cell apoptosis. Higher positive (negative) correlation corresponding to darker blue (red). "×" in the figure meaning the *P*>0.05.

96365 under hypoxic conditions ($P<0.05$), compared to the HYP group. However, caspase3 mRNA expression was significantly increased with treatment of FFA under hypoxic conditions ($P<0.05$) (Fig. 4F). After cells were under hypoxic conditions and treatment with FFA for 48 h, caspase3 mRNA expression levels remained unchanged ($P>0.05$) (Fig. 4F). This data indicated that TRPC6 plays a critical role in PASMC proliferation and apoptosis. Inhibition of TRPC6 reduced cell proliferation and induced apoptosis, while activation of TRPC6 promoted proliferation and decreased apoptosis in hypoxic PASMCs.

Intracellular Ca^{2+} levels, TRPC6 levels, proliferation and apoptosis correlated in hypoxia exposed PASMCs: Pearson correlation analysis was performed to establish the relationship between intracellular Ca^{2+} levels, TRPC6 levels, cell proliferation and cell apoptosis. As shown in Fig. 5A, intercellular Ca^{2+} levels positively correlated with TRPC6 mRNA levels. In addition, TRPC6 mRNA levels highly correlated with the number of cells in the S phase. However, TRPC6 mRNA levels negatively correlated with cells in the G2/M phase, but showed a weak correlation with the ratio of cells in the G0/G1 phase. The ratio of early and all apoptotic cells negatively correlated with TRPC6 mRNA levels, but the ratio of late apoptotic cells and TRPC6 mRNA levels did not show a significant correlation (Fig. 5B). These results indicated that intracellular Ca^{2+} levels, TRPC6 mRNA levels, proliferation and apoptosis may be correlated in hypoxia exposed PASMCs.

DISCUSSION

Exuberant pulmonary vascular remodeling caused by an imbalance between proliferation and apoptosis in PASMC is considered a vital process involved in pulmonary arterial hypertension (Chen *et al.*, 2018). Several studies found that intracellular Ca^{2+} levels mediated by TRPC6 are important in hypoxia-induced PASMC proliferation in mammals (Du *et al.*, 2017). However, the roles of TRPC6 in PASMCs of broilers is currently unknown. In this study, we focused on the role of TRPC6 in hypoxia-induced proliferation and apoptotic suppression of chicken PASMCs. Our data suggests that hypoxia-induced TRPC6 activation increased intracellular Ca^{2+} levels, promoted proliferation and inhibited apoptosis of chicken PASMCs.

Intracellular Ca^{2+} levels have three main functions in the vasculature. First, intracellular Ca^{2+} levels in vascular smooth muscle cells participated in excitation-contraction coupling, which is critical for sustained vascular contractions (Ma *et al.*, 2016). Second, changes in intracellular Ca^{2+} levels caused by endogenous and exogenous factors affect cell cycle progression (Machaca, 2011). Finally, calcium ions affect the contraction and proliferation of cells by indirectly regulating the activity of nuclear transcription factors. In this study, we found that intracellular Ca^{2+} levels in PASMCs were significantly increased when cells were cultured under hypoxic conditions for 48h, but that intracellular Ca^{2+} levels remained unchanged when cells were exposed to hypoxic conditions for 24h. Parpaite *et al.* (2016) found that increased intracellular Ca^{2+} levels were not observed when rat PASMCs were cultured in hypoxic conditions for 24h. In addition, hypoxia accelerated the conversion of chicken PASMCs from G0/G1 to S phases in hypoxic conditions at 48h. Previous study using Hela cells suggested that store-operated Ca^{2+} entry promotes the cell cycle transformation from the G1 to S phases (Chen *et al.*, 2018). Therefore, we speculated that the increase in intracellular Ca^{2+} concentrations caused by hypoxia may be an important factor to promote cell cycle conversion in chicken PASMCs. In this study, we also found that apoptosis was significantly decreased in hypoxic conditions at 48h. Previous studies have shown that hypoxia reduces proliferation of mammalian PASMCs (Gui *et al.*, 2017). These findings are consistent with our findings. Therefore, hypoxia appears to influence intracellular Ca^{2+} levels, proliferation, apoptosis and the cell cycle of chicken PASMCs.

In this study, we found that decreased levels of TRPC6 using SKF 96365 in hypoxic PASMCs reduced intracellular Ca^{2+} levels and expression of TRPC6 mRNA. Overexpression of TRPC6 using FFA in hypoxic PASMCs increased TRPC6 mRNA expression levels. Moreover, inhibition of TRPC6 attenuated proliferation of hypoxic PASMCs by causing cell cycle arrest in the G2/M phase. A previous study showed that activation of TRPC6 intensifies proliferation of hypoxic human PASMCs by causing cell cycle arrest in the S phase (Han *et al.*, 2018). However, inhibition of TRPC6 attenuated increased Ca^{2+} levels, suppressed cell growth and arrested the cell cycle in the G2/M phase in cancer cells (Song *et al.*, 2013). This research is consistent with our findings. Moreover, many studies indicated that TRPC6 was involved in apoptosis (Yao *et al.*, 2016). It was found that decreased expression of TRPC6 triggered apoptosis, but increased expression of TRPC6 enhanced apoptosis of primary PASMCs in chickens. At the same time, repression of TRPC6 decreased caspase3 mRNA levels in hypoxic PASMCs, but increased levels of TRPC6 also increased caspase3 mRNA levels. However, intracellular Ca^{2+} levels, cell cycle arrest and apoptosis were not significantly changed when hypoxic PASMCs were treated with SKF 96365 or FFA at 48 h. *In vitro* experiments, cells were incubated with SKF 96365 or FFA for 24 hours (He *et al.*, 2017). In these experiments, both SKF 96365 and FFA showed obvious effects, which showed significant effects on cell proliferation, apoptosis and intracellular Ca^{2+} levels. However, in a study investigating the effects of TRPC6 on the proliferation of intervertebral disc cells, SKF was co-incubated with intervertebral disc cells for 5 days under microgravity (Franco-Obregon *et al.*, 2018). Researchers found that SKF treatment significantly affected cell cycle distribution, but the effects were not significant after 3 days of microgravity treatment (Franco-Obregon *et al.*, 2018). We suspected that although FFA and SKF 96365 are excellent pharmacological tools for studying TRPC6, the effects diminished as the drug were metabolized. Nevertheless, through Pearson correlation analysis, we found that TRPC6 mRNA expression levels were significantly and positively correlated with intracellular Ca^{2+} levels after hypoxic PASMC treatment with SKF or FFA.

Conclusions: We propose that hypoxia promoted proliferation and reduced apoptosis in chicken PASMCs through TRPC6 mediation of intracellular Ca^{2+} levels. TRPC6 is a promising target for the treatment of AS. This study suggests that TRPC6 plays a significant role in hypoxia-induced proliferation and apoptosis of chicken PASMCs. Therefore, further studies are required to elucidate the functions of TRPC6 in the proliferation of hypoxic PASMCs and the search for a new drug that inhibits TRPC6.

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Authors contribution: Conceived and designed the experiments: NQ, JP, ZT and YL. Performed the experiments: NQ, JP, HC, ZK, CP, BL, and QZ. Analyzed the data: KM, RMB, NQ, JP and HC. Contributed reagents/materials/analysis tools: NQ, JP, ZT and YL. Wrote the paper: NQ and JP. NQ, JP, KM, RHP and Q handled the revision.

REFERENCES

Chen FZ, Wang H, Lai JD, *et al.,* 2018. 3-Bromopyruvate reverses hypoxia-induced pulmonary arterial hypertension through inhibiting glycolysis: In vitro and in vivo studies. Int J Cardiol 266: 236-41.

Du Y, Fu J, Yao L, *et al.,* 2017. Altered expression of PPARgamma and TRPC in neonatal rats with persistent pulmonary hypertension. Mol Med Rep 16:1117-24.

Franco-Obregon A, Cambria E, Greutert H, *et al.,* 2018. TRPC6 in simulated microgravity of intervertebral disc cells. Eur Spine J 27:2621-30.

Gui D, Cui Z, Zhang L, *et al.,* 2017. Salidroside attenuates hypoxia-induced pulmonary arterial smooth muscle cell proliferation and apoptosis resistance by upregulating autophagy through the AMPK-mTOR-ULK1 pathway. BMC Pulm Med 17:191.

Guignabert C and Dorfmuller P, 2013. Pathology and pathobiology of pulmonary hypertension. Semin Respir Crit Care Med 34:551-9.

Han JL, Tian HY, Liu Y *et al.,* 2018. 5-HT promotes pulmonary arterial smooth muscle cell proliferation through the TRPC channel. Cell Mol Biol 64:89-96.

He X, Li S, Liu B, *et al.,* 2017. Major contribution of the 3/6/7 class of TRPC channels to myocardial ischemia/reperfusion and cellular hypoxia/ reoxygenation injuries. Proc Natl Acad Sci USA 114:E4582-91.

Kamely M, Torshizi MAK and Rahimi S, 2015. Incidence of ascites syndrome and related hematological response in short-term feed-restricted broilers raised at low ambient temperature. Poult Sci 94:2247-56.

Liao J, Yang F, Chen H, *et al.,* 2019. Effects of copper on oxidative stress and autophagy in hypothalamus of broilers. Ecotoxicol Environ Saf 185:109710.

Ma R, Chaudhari S, and Li W, 2016. Canonical Transient Receptor Potential 6 Channel: A New Target of Reactive Oxygen Species in Renal Physiology and Pathology. Antioxid Redox Signal 25:732-48.

Machaca K, 2011. Ca(2+) signaling, genes and the cell cycle. Cell Calcium 49:323-30.

Parpaite T, Cardouat G, Mauroux M, *et al.,* 2016. Effect of hypoxia on TRPV1 and TRPV4 channels in rat pulmonary arterial smooth muscle cells. Pflugers Arch 468:111-30.

Riley CM and Sciurba FC, 2019. Diagnosis and Outpatient Management of Chronic Obstructive Pulmonary Disease: A Review. JAMA 321:786-97.

Song JY, Wang Y, Li X, *et al.,* 2013. Critical role of TRPC6 channels in the development of human renal cell carcinoma. Mol Biol Reports 40:5115-22.

Sylvester JT, Shimoda LA, Aaronson PI, *et al.,* 2012. Hypoxic pulmonary vasoconstriction. Physiol Rev 92:367-520.

Tan X, Shao FJ, Fan GJ *et al.,* 2018. Expression of angiogenic factors and plexiform lesions in the lungs of broiler and layer chickens: A comparison. Poult Sci 97:1526-35.

Voelkel NF and Tuder RM, 2000. Hypoxia-induced pulmonary vascular remodeling: a model for what human disease? J Clin Invest 106:733-8.

Xia Y, Yang XR, Fu Z, *et al.,* 2014. Classical transient receptor potential 1 and 6 contribute to hypoxic pulmonary hypertension through differential regulation of pulmonary vascular functions. Hypertension 63:173-80.

Yang Y, Sun F, Zhang C, *et al.,* 2013. Hypoxia promotes cell proliferation by modulating E2F1 in chicken pulmonary arterial smooth muscle cells. J Anim Sci Biotechnol 4.

Yao XM, Liu YJ, Wang YM, *et al.,* 2016. Astragaloside IV prevents high glucose-induced podocyte apoptosis via downregulation of TRPC6. Mol Med Reports 13:5149-56.

Zhang H, Chang Z, Mehmood K, *et al.,* 2019. Calcium-Sensing Receptor Arbitrates Hypoxia-Induced Proliferation of Pulmonary Artery Smooth Muscle Cells via the G Protein-PLC-IP3 Pathway. Crit Rev Eukaryot Gene Expr 29:69-76.

Zhang H, Chang Z, Mehmood K, *et al.,* 2018. Tetramethylpyrazine inhibited hypoxia-induced expression of calcium-sensing receptors in pulmonary artery smooth muscle cells in chickens. J Biol Regul Homeost Agents 32:489-95.