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Full Length Article

Hypoxia Promotes VEGF and HIF-1α Expressions in Endometrial Epithelium Cells of Yaks (*Bos grunniens*) in Qinghai-Tibet Plateau

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Abstract

Hypoxia occurs during the early stages of pregnancy that triggers angiogenesis by promoting vascular endothelial growth factor (VEGF) and hypoxia inducible factor (HIF)-1α expressions. Currently, there is limited information regarding VEGF and HIF-1α expression in endometrial epithelium cells of yaks. The in-vitro study was designed to check the expression of VEGF and HIF-1α in endometrial epithelium cells in experimentally induced hypoxia cells and normal cells and the effects of hypoxia on the regulation of pregnancy in endometrial epithelium cells in yaks. Primary cell culture and subculture was performed from the endometrial epithelium cells to assess the gene profile by using immunofluorescence, RT-qPCR and Western-blotting analysis. Results showed that VEGF and HIF-1α mRNA and protein expression levels were significantly increased in experimentally induced hypoxia culture cells as compared with normal cells. These results suggest that hypoxia plays an important role in the over expression of VEGF and HIF-1α gene in endometrial epithelium cells of yaks. So, it is concluded that hypoxia aggravate angiogenesis to promote the supplement of oxygen, nutrients, and the exchange of metabolites between the mother and the fetus in endometrial epithelium cells via regulations of HIF-1α/VEGF signaling pathway in Yaks.

Keywords: Pregnancy; Yak; Endometrial epithelium cell;

Introduction

In the early stages of pregnancy, oxygen is the necessary substance for the normal functioning of placenta and fetal growth in late pregnancy (Dimasuay *et al.*, 2016; Pouyssegur and López-barneo, 2016; Fajersztajn and Veras, 2017). The spiral arteriolar blockage by endovascular cytotrophoblasts (CTBs) in matrix causes no maternal blood flows into the intervillousspace (IVS) that make the development of early placenta and embryo in relatively low oxygen environment (Petty *et al.*, 2007; Pouyssegur and López-barneo, 2016; Fajersztajn and Veras, 2017). After 2 to 4 weeks of gestation, the special syncytial formed in the placenta place with urine chorionic disintegration and a large area of vascular formed, the connection between majority of chorionic and the womb have been successfully established that maternal blood flow began to provide the gestation nutrition and gas exchange between the mother and the fetus (Jr, 1965). Hypoxia is an important regulatory factor related to the morphological and functional changes of placenta. In normal pregnancy, the hypoxic environment of the placenta is an essential physiological procedure during the first trimester (Pouyssegur and López-barneo, 2016; Fajersztajn and Veras, 2017).

Hypoxia injury is a common pathological process in many diseases and one of the most important reasons that causes death (Darby and Hewitson, 2016). With the rapid development of molecular biology and cell detection technology, the research of hypoxia injury and its adaptation mechanism is becoming more and more advanced (Lokmic *et al.*, 2012; Darby and Hewitson, 2016). Hypoxia is a potential lethal factor for cell growth that affect the cell cycle, morphological structure, metabolism, signaling pathway, proliferation, differentiation and apoptosis (Fernandes *et al.*, 2010; Li *et al.*, 2017). HIF-1α is core adjusting factor to the body in response to hypoxia induced gene expression and for restoration of the environment in the cells (Gardner and Corn, 2008; Kaluz *et al.*, 2008). This process will promote the supply of oxygen, reduce the cell oxygen demand and protect itself from damage.

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The VEGF is a common factor that promotes angiogenesis. The body can secrete a large amount of VEGF with the stimulation of hypoxia, inflammatory factor, estrogen and formation of vessels (Baranova *et al.*, 2007; Abe, 2008; Riddell *et al.*, 2012). Under hypoxia, HIF-1α increase the generation of red blood cells and blood capillary hyperplasia via mediate the expression of VEGF to improve the oxygen supplement. On other hand, HIF-1α enhances hypoxia adaptation tolerance through mediating the expression of glucose transporter and glycolytic enzyme (Critchley *et al.*, 2006).

Yak (*Bos grunniens*) is relatively an ancient original indigenous breed with the characteristic of a longhaired animal found throughout the Qinghai-Tibet plateau at 3000−5000 m high altitude of China (Li *et al.*, 2014). Yak is a rare high altitude pasture breed in China, but due to the long-term natural selection in a particular ecological environment in the plateau, the yak has adopted the special biological characteristics of strong survival ability, physique and resistance to disease and bad weather conditions such as low pressure, hypoxia and cold (Li *et al.*, 2014; Ma *et al.*, 2016). Because of its unique plateau adaptability, yak is a good model for studying hypoxia physiology mechanism (Yu *et al.*, 2017). To date little information's is available regarding hypoxia on the regulation of pregnancy in yaks. Therefore, the present study was conducted to assess the effect of hypoxia on regulation of VEGF and HIF-1α expressions in endometrial epithelium cells.

Materials and Methods

All the experiments were performed as per guidelines of Agriculture and Animal Husbandry University Animal Care Committee and performed in accordance with the international guidelines for animal welfare.

Chemicals and Reagents

The DMEM-F 12 media with 2.5 mM L-glutamine (Cat No. SH30023.01B; HyClone Laboratories Beijing, China), while Fetal bovine serum (FBS) (Lot: 1414426; gibco, Australia) and scorbic acid (Catalog# 0764 Armesco) were used during the experiment. Remaining chemicals were purchased from Service bio Co. Ltd Wuhan, China.

Cultivation of Endometrial Epithelium Cells from Yaks

The tissue fragments were digested with collagenase II for 2 h and transferred to the culture bottle, the endometrial epithelium cells were observed and adherent among primary cultured cells for 12 h.

Cell Culture

The endometrial epithelium cells were cultured in DMEM-F12 media containing 10% FBS, 50 U/mL of penicillin, streptomycin and ascorbic acid. The cells were divided into two groups: normal group, hypoxia group and then incubated at 37°C for 24−48 h with 5% CO_2.

Identification of Endometrial Epithelium Cells

Immunohistochemistry was performed using a specific antibody against cytokeratin 18 (CK18), an epithelial cell marker. First, cells were transferred onto glass cover slips and then fixed in ice-cold acetone at room temperature for 10 min. Endogenous peroxidase activity was blocked by hydrogen peroxide, and non-specific antibody binding was blocked by incubation with 5% bovine serum albumin for 20 min at room temperature. Then, the cells were incubated with a CK18 antibody at 37°C for 1 h, followed by a second antibody at 37°C for 20 min. Images of the cells were obtained under a microscope.

Immunofluorescence Assay

Immunofluorescence was performed using the specific antibody against VEGF (Service bio, GB11034) and HIF-1α (Ruiying, RLT2133) according to previous study with minor modifications (Zhang *et al.*, 2017). Firstly, the cells were cultured in 6-well plates and sections of cells were incubated with the antibody for VEGF and HIF-1α overnight at 4°C. After that incubated with secondary antibody in the dark for 2 h at room temperature and then VEGF and HIF-1α proteins were mounted for nuclear counterstaining. The images of the cells were obtained under a microscope.

RT-qPCR Assay

Total RNA was extracted from the cell samples by using the Trizol (Invitrogen, USA) (Chang *et al.*, 2017; Zhang *et al.*, 2017). A total of 20 µL cDNA was synthesized by cDNA kit (Tian Gen, China). The RT-qPCR was performed in quadruplex with Step One-Plus™ Real-Time PCR System (Applied Biosystems, CA, USA). GAPDH primers were selected as reference gene, whereas results were expressed by$2^{-\Delta\Delta Ct}$ comparative method.

Western-blotting Analysis

The proteins from cells were extracted using RIPA lysis solution for 30 min. The protein concentration was calculated using BCA kit. Then the equal amounts of protein were subjected to 10% SDS polyacrylamide gel and transferred onto PVDF membrane. The membrane was incubated with primary antibody at 4°C overnight. The membranes were washed 3 times with TBST (tris-buffered saline containing 0.1% Tween 2.0) for 5 min each then incubated with a secondary antibody for 1 h at room temperature. After washing, the image was taken with an imaging system and β-actin used as an internal standard during the study.

Statistical Analysis

The data were checked with one way ANOVA and student t-test to compare the differences by using SPSS 19.0 software. $P<0.05$ was statistically significant.

Results

After 36 h of culture, a monolayer of cells were digested and translated to the new bottle for culture and after a monolayer of cells began to form, it exhibited a typical cobblestone appearance with tightly packed cells (Fig. 1).

Cell Identification

The CK18 immunohistochemical identification of endometrial epithelium cells was used in this study for the identification of cells. The immunohistochemistry results showed that antibodies were localized and positively stained (Fig. 2).

Immunofluorescence Evaluation of VEGF and HIF-1α Protein in the Endometrial Epithelium Cells

The expression of VEGF and HIF-1α was confirmed in endometrial epithelium cells by using immunofluorescence technique and observed with a light microscope. The VEGF and HIF-1α were highly expressed (apparent positive reaction) in hypoxia group as compared to normal group (Fig. 3).

VEGF and HIF-1α Expression Levels in Normal and Hypoxia Group

The expression of VEGF and HIF-1α genein endometrial epithelium cells was checked in normal and hypoxia groups. The results showed that the expression of both genes were significantly up-regulated in hypoxia group compared to normal group as shown in Fig. 4.

Similarly, the western blotting analysis results were parallel to gene expression of VEGF and HIF-1α. The western blot analysis depicted the expression level of VEGF and HIF-1α was significantly higher in hypoxia group than normal group ($P < 0.01$) as shown in Fig. 5 and 6.

Discussion

The pregnancy is a series of complicated processes for mammal (Woelk *et al.*, 2010). The most important parts of pregnancy are the formation of the placenta, and the failure of the placenta formation process leads to the failure of pregnancy, which is a big issue in the mammal breeding industry (Sammin *et al.*, 2009). There are important changes in the blood flow of uterus during the formation of the placenta, so that the oxygen environment of the placenta is also changed.

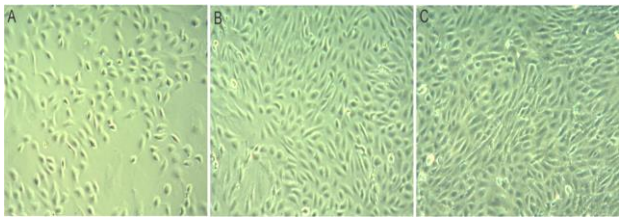

Fig. 1: The culture of endometrial epithelium cells (A, after 12 h; B, after 24 h; C, after 36 h)

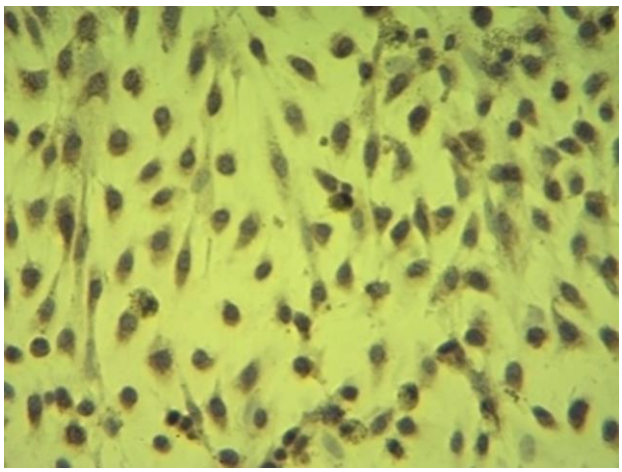

Fig. 2: The CK18 immunohistochemical identify the endometrial epithelium cells

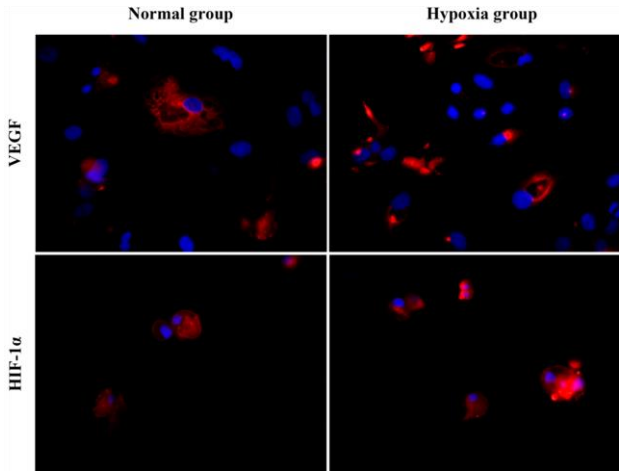

Fig. 3: Expression pattern of VEGF and HIF-1α by immunofluorescence analysis in endometrial epithelium cells

It indicates that oxygen is key adjustment factor during the growth of placenta (Carter, 2015). Almost, 90% of the world yak population is in Tibet and these are economically important animals for Tibetans. So, any yak pregnancy failure is a serious threat for reproduction rate and economic losses to yak population (Li *et al.*, 2014).

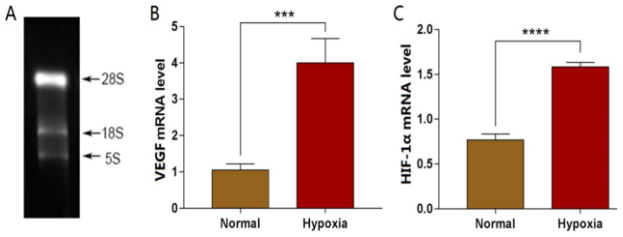

Fig. 4: The real-time quantitative PCR analysis of VEGF and HIF-1α in endometrial epithelium cells. (A) The RNA were run on a 1.5% denaturing agarose gel, the 5S, 18S and 28S ribosomal RNA bands are clearly visible (B) mRNA levels of VEGF was detected by RT-qPCR; (C) mRNA levels of HIF-1α was detected by RT-qPCR. ***P-value < 0.001; ****P-value < 0.0001 normal versus hypoxia

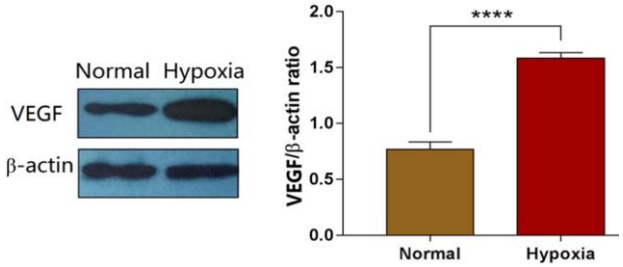

Fig. 5: The protein level of VEGF in endometrial epithelium cells between normal and hypoxia group. β-actin was used as the control. The values are presented as the means ± S.E.M (n=5). ****P-value <0.0001 normal versus hypoxia

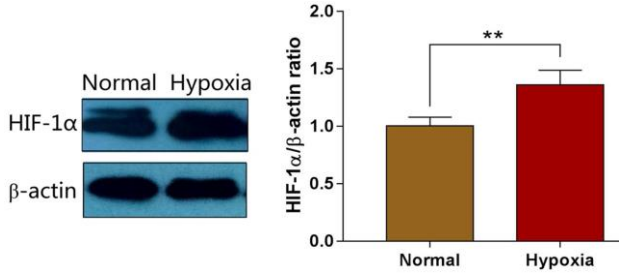

Fig. 6: HIF-1α protein level was analyzed in endometrial epithelium cells. The β-actin was used as the control. Data expressed in arbitrary units as the means ± S.E.M. (n=5). **P-value <0.01 normal versus hypoxia

The formation of embryos is important during pregnancy; the difficulties of early embryo implantation are the main causes of yak infertility. The early embryos implantation requires a synchronized development of womb (Binder *et al.*, 2014). The womb in the early stages is under the hypoxia condition; therefore, oxygen-deficient environment in early pregnancy may play an unusual role in the formation of placenta (Petty *et al.*, 2007; Pringle *et al.*, 2010). During remodeling and growth of placenta, the embryo is in anoxic environment. By means of continuous invasion of trophoblast cells, the vascular network of placental is gradually established to ensure the supply of oxygen, nutrients and metabolites to embryo. There are many angiogenesis promoting factors including VEGF, EGF, TGF-β, bFGF. The VEGF is the most important vascular growth factors that promotes angiogenesis and enhance vascular permeability (Osadnik *et al.*, 2016).

HIF-1α increase the generation of red blood cells and blood capillary hyperplasia mediate the expression of VEGF, so it increases the supplement of oxygen in endometrial epithelium cells (Helske *et al.*, 2001). HIF-1α can enhance hypoxia adaptation tolerance during hypoxia via mediated the expression of glucose transporter and glycolytic enzyme (Critchley *et al.*, 2006; Baranova *et al.*, 2007). This may be a response to chronic hypoxia vascular adaptation in the mother-fetal. Therefore, hif-1α regulates the development of placenta between normal and pathological pregnancy. HIF-1α is major regulator of hypoxic responses, such as endometrial epithelium cells growth arrest, survival, maturation, and apoptosis that are essential for uterus (Helske *et al.*, 2001).

Recent studies showed that the production of VEGF in endometrial epithelium cells is regulated by hypoxia and HIF-1α, which play major roles in the first trimester of pregnancy (Dai *et al.*, 2015). Our finding showed that the expression of VEGF and HIF-1α in endometrial epithelium cells were significant increase in hypoxia group as compared with normal group.

Conclusion

Hypoxia has effective performance on endometrial epithelium cells in the first trimester of pregnancy to ensure a normal gestation environment in Yaks. The present study demonstrated that hypoxia aggravate angiogenesis to promote the supplement of oxygen, nutrients, and the exchange of metabolites between the mother and the fetus in endometrial epithelium cells via regulations of HIF-1α/VEGF signaling pathway in Yaks.

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