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Original Article

The effect of androgen on wool follicles and keratin production in Hetian sheep

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(With 8 figures)

Abstract

To investigate the optimal androgen concentration for culturing Hetian sheep wool follicle and to detect effects of androgen concentration on wool follicle cell proliferation and apoptosis using immunofluorescence labeling and real-time quantitative fluorescence determinations of wool keratin-associated protein gene expression levels. Wool follicles were isolated by microdissection and wool follicles and skin pieces were cultured in various concentrations of dihydrotestosterone (DHT) in culture medium. Next, daily lengthwise growth measurements of wool follicles were obtained using a microscopic micrometer. Cultured Hetian wool follicles were stained using the SACPIC method to reveal wool follicle structure, while sheep skin slices were used to observe cell proliferation by immunostaining and cell apoptosis using the TUNEL method. At the molecular biological level, keratin-associated protein (*Kap*) gene expression was studied using wool follicles cultured for various numbers of days *in vitro*. Effects of androgen concentrations on Hetian wool follicle growth and development were experimentally studied. EdU proliferation assays revealed that androgen promoted cell proliferation within wool follicle dermal papillae. TUNEL apoptosis detection demonstrated that androgen treatment could delay cell apoptosis. Quantitative reverse transcription polymerase chain reaction (qPCR) results demonstrated that gene expression level patterns of Hetian mountain sheep super-high sulfur protein. *Kap*1.1*, KIF*1.2*, Kap*2.12 and *Kap*4.2 gene expression level of the mountainous experimental group was significantly higher than plains Hetian sheep. An androgen concentration of 100 nM can promote the growth of Hetian wool follicle cells *in vitro*, resulting in overexpression of some genes of the *Kap* family.

Keywords: androgen, dihydrotestosterone, Hetian sheep, wool follicle, Keratin- associated protein.

Efeito do androgênio na produção de folículos e queratina em ovinos Hetianos

Resumo

Investigar a concentração ideal de andrógenos em cultura de folículos pilosos de carneiro Hetiano e detectar os efeitos da concentração de andrógenos na proliferação e apoptose de células foliculares, por meio de imunofluorescência e de determinação quantitativa, em tempo real, da fluorescência dos níveis de expressão gênica de proteína associada à queratina. Folículos pilosos foram isolados por microdissecção, e folículos de lã e pedaços de pele foram cultivados em várias concentrações de di-hidrotestosterona (DHT) em meio de cultura. Em seguida, medições diárias de crescimento longitudinal dos folículos capilares foram obtidas usando um micrômetro microscópico. Folículos de lã cultivados de Hetianos foram corados pelo método SACPIC para revelar a estrutura do folículo piloso, enquanto fatias de pele de carneiro foram usadas para observar a proliferação celular por imunocoloração e apoptose celular por meio do método TUNEL. Em âmbito da biologia molecular, a expressão gênica da proteína associada à queratina (Kap) foi estudada usando folículos capilares cultivados por vários dias, in vitro. Os efeitos das concentrações de andrógenos no crescimento e desenvolvimento dos folículos de lã de Hetianos foram estudados experimentalmente. Ensaios de proliferação de EdU revelaram que o andrógeno promoveu a proliferação celular dentro das papilas dérmicas do folículo piloso. A detecção de apoptose por TUNEL demonstrou que o tratamento com andrógeno poderia atrasar a apoptose celular. Os resultados da reação em cadeia da polimerase transcrição reversa quantitativa (qPCR) demonstraram que os padrões de expressão gênica da proteína de enxofre Kap1.1, KIF1.2, Kap2.12 e Kap4.2 foram significativamente maiores no grupo de ovinos Hetianos de montanha. Uma concentração de androgênio de 100 nM pode promover o crescimento de células foliculares de lã de Hetianos in vitro, resultando na superexpressão de alguns genes da família Kap.

Palavras-chave: androgênio, dihydrotestosterone, Hetiano ardiak, folículo de carneiro, Keratinari lotutako proteina.

1. Introduction

Hetian sheep are a breed of sheep that have been cultivated by farmers and herdsman in Hetian, Xinjiang, China for centuries. The breed is famous for its ability to adapt to geographically unique desert and semi-desert environments and for its ability produce high-quality wool for production of semi-coarse wool carpets in China. Moreover, secondary types of wool and fake wool that are continuously being integrated into the current Hetian wool market have resulted in a decline in Hetian carpet quality. Studies have shown that growth and development of wool follicles are regulated by many factors, including regulation at the hormone level. one of the factors is Androgens, a major regulator of human hair growth, act on hair follicle epithelial cells to influence follicle growth and the biological hair growth cycle (Wang et al., 2009) . Recent studies have indicated that administration of androgen to the skin of rats can both significantly accelerate development of hair follicles and prompt them to enter the growth phase, thus functioning as an endogenous secretory regulator of the hair cycle (Scher and Sawyers, 2005). and the AR (androgen receptor) -ligand complexes ultimately bind to DNA to activate hormone-responsive components that regulate gene expression and affect protein synthesis such as Keratin association protein (*Kap*)and Keratin Intermediate filament(IF). As the effects of androgens on hair follicles are still unknown, the purpose of this study is to explore androgen effects on follicle growth of keratin-associated protein gene expression levels and the hair follicle cell development (Lee et al., 2006). This study should provide a theoretical foundation on which to base future efforts toward improving Hetian wool quality and regulate hair follicle development.

2. Material and Methods

2.1. Material

2.1.1. Hetian sheep of Xinjiang

Hetian sheep were purchased from suppliers in two counties in the Hetian Region of Xinjiang Province, China. Sheep that were studied included mountain and plain type of Hetian sheep varieties. The mountain variety was purchased in Cele County and the plains variety was purchased in Luopu County. Sheep were raised in the experimental animal feeding center of the College of Animal Science, Tarim University. Regular testing of sheep was conducted to ensure that they were healthy enough to meet experimental requirements (Liu et al., 2014).

2.1.2. Main reagents

KeyFluor 488 Click-iT® EdU Imaging Test Kit (kga31-50, Nanjing Kaiji Biotechnology Co., Ltd., China), anti-rabbit IgG secondary antibody (sa00003-2, Wuhan Sanying Biotechnology Co., Ltd., China), OCT cryopreservation agent (Leica Biosystems Inc., Germany), HistoChoice transparent tissue fixative and bovine serum albumin (BSA) (Gibco, Inc., USA), anti-fluorescence quenching agent (Beijing Solab Technology Co., Ltd., China), phosphate buffer (DPBS, Beijing Unikang Biotechnology Co., Ltd., China), 4% polyformaldehyde and hematoxylin (Beijing Berlin Biological Technology Co., Ltd., China), Safranin O solution (Beijing Solaibao Biological Technology Co., Ltd., China), Oligo (dT) 12-18 Primer, 2× Power Taq PCR Master Mix, DL2000 DNA Marker (TaKaRa Dalian, China), agarose, SYBR Green I dye were used in this work with other routine laboratory reagents.

2.1.3. Main instruments

The main instruments used in this work included the following: semi-automatic tissue hydroextractor, semi-automatic paraffin sectioning machine module, semi-automatic tissue embedding machine, biological tissue stand (Leica, Germany); low temperature high-speed refrigerated centrifuge (USA); gel imaging system (China), gradient PCR system (USA), real-time fluorescent quantitative PCR system (China), surgical dissection microscope (Germany), inverted fluorescence microscope (Zeiss, Germany), McAudi Visual Microscope (China).

2.2. Experimental methods

First, we used a microdissection instrument to isolate wool follicles from the undersides of ventral skin samples taken from plains and mountain types of Hetian sheep. Triplicate skin samples, each with diameter of 1 cm, were stripped of their wool coat layers at an ultraclean workbench. Each sample was wiped twice with 75% ethanol, then washed three times in succession with PBS for 3 min/wash. Skin samples cut into 3 mm × 8 mm-size pieces were processed to remove subcutaneous adipose tissue (Driskell et al., 2011), then placed into 25-mm diameter sterile plastic culture dishes. Next, 4 ml of 5% FBS + DMEM with added streptomycin and cell proliferation assay culture medium containing 20 μM EdU and androgen at concentrations of 1000 nM, 100 nM, 10 nM and 0 nM (negative control group without androgen) was added to dishes. Each dish containing about 20 follicles was immediately placed in a humidified incubator at 37 °C with 5% CO_2 and incubated for 6 d in triplicate. Triplicate samples were removed every 2 d and some samples were prepared for microscopy using paraffin embedding agent, while others were stored frozen at -80 °C for qRT-PCR or sectioned using a cryostat to generate 8-μm thick slices. A microscope was used to observe and measure the growth length of each wool follicle daily and results were analyzed for statistical significance.

Next, triplicate samples of Hetian sheepskin samples after treatments with various androgen concentrations were fixed in 4% paraformaldehyde for 24 h then trimmed at a microdissection workbench to create tissue blocks. Tissue blocks were soaked in deionized water in a paraffin embedding box for 5 h to remove impurities. Next, samples were processed using a semi-automatic tissue hydroextractor and established procedures to perform sample dehydration, sample fixation with transparent fixative

and sample embedding in paraffin (Johnston et al., 2016). A semi-automatic tissue embedding machine was used to process samples of wool follicles cut longitudinally. First, dehydrated samples were placed in a metal paraffin embedding box. Process settings were as follows: 70% ethanol for 2 h, 80% ethanol for 2 h, 90% ethanol for 30 min, 95% ethanol for 1 h, anhydrous ethanol for 1 h, anhydrous ethanol for 1 h, HistoClear/ethanol (1:1) for 1 h, HistoClear for 2 h, paraffin I at 58 °C for 2 h, paraffin II at 60 °C for 2 h. Next, sample fixation was performed followed by hot paraffin embedding at 55°C. After solidification and trimming of paraffin blocks containing specimens, blocks were sectioned using a paraffin cutting machine (Leica) to generate 8 µm thick slices which were stored at RT until needed.

In the third step, SACPIC staining and immunofluorescent staining of paraffin sections were conducted. For the SACPIC dyeing process, paraffin sections were immersed in HistoClear for 5 min, then rinsed with 80% ethanol for 30 s, 30% ethanol for 10 s and PBS for 10 s. Next, hematoxylin staining was performed for 4 min followed by a PBS rinse for 10 s, 70% ethanol elution for 20 s, a safranin dye step for 5 min, 70% ethanol elution for 20 s, anhydrous ethanol rinse for 10 s, picric acid staining for 30 s and a PBS rinse for 30 s. Finally, sections were dyed with picric acid-indigo carmine dye for 2 min, followed by 75% ethanol elution for 20 s and an anhydrous ethanol rinse for 10 s. Samples were preserved in neutral resin at room temperature after drying then were photographed under a light microscope to observe their tissue structure. Meanwhile, before immunofluorescence staining we performed EdU staining. First, an EdU-labeled pre-cut paraffin section was dewaxed and rehydrated then 1 ml of 4% paraformaldehyde was added to fix each sample at room temperature for 30 min. Next, 3% bovine serum albumin solution in PBS was used to wash the specimen twice for 5 min/wash then 0.5% Triton X-100 in PBS was added to allow the fluorescent probe to permeate cell cytoplasmic and nuclear membranes after 20 min. Next, each specimen was washed twice with 3% FBS serum for 5 min/wash to remove Triton X-100 then each specimen was flooded for 1 s with 0.5 ml of KeyFluor 488 Click-iT® response mixture (as per kit instructions) and incubated for 30 min at room temperature under humid conditions without light. A fluorescently-labeled negative control was prepared in parallel using PBS instead of KeyFluor 488 Click-iT® response mixture. Next, specimen dewaxing and rehydration were carried out then 100 µl Triton X-100 in PBS serving as a 0.1% cell permeabilization solution was added to the surface of each section followed by incubation at room temperature for 5 min. Next, 50 µl of Labeling Solution containing TdT was prepared and 2 µl was added to the surface of each slice followed by incubation at 37 °C in the dark for 1 h. PBS was used in place of Labeling Solution for the negative control group. Next, each specimen received 100 µl of Triton X-100 added dropwise then each sample was allowed to stand at room temperature for 5 min followed by careful removal of surrounding liquid by suctioning. Steps from the Triton X-100 addition to suctioning of liquid were repeated 3 times. Next, 2.5 µl of anti-fluorescence quenching agent was added and a cover glass was applied to seal each slide. Slides were taken to the darkroom and a Zeiss inverted visual fluorescence microscope was used to observe and photograph the slides.

Next, samples of Hetian sheepskin were taken from the -80 °C freezer. Each sample (~80-100 mg) was used to prepare total RNA. RNA concentrations were determined from A_{260} and A_{280} readings measured using a spectrophotometer, with A_{260}/A_{280} values of 1.8-2.0 reflecting suitable RNA purity. A One-Step gDNA Removal and cDNA synthesis SuperMix Reverse Transcription RNA Kit was used to obtain cDNA from total RNA. RT-PCR 18S ribosomal DNA primers were used to verify cDNA quality (Innis et al., 1990). The cDNA concentration was adjusted to 250 ng/ml. Primers for quantitative real-time PCR analysis of *Kap1.1*, *KIF1.2*, *Kap2.12*, *Kap 4.2* gene expression and 18S ribosomal DNA primers were synthesized by Sangon Bio-engineering Co., Ltd. (Shanghai). Primer sequences are shown in Table 1. For quantitative real-time PCR, 3 replicates were performed for each sample. Relative mRNA content between compared samples was calculated using the $2^{-\triangle\triangle Ct}$ method (Brankin et al., 2010), where Ct = (target

Table 1. Oligonucleotide sequences.

Gene name	Primer sequences (5′→3′)	Fragment length
*Kap*1.1	F:5′-CTTAAGAATGAAGGGGGAAGC-3′ R:5′-ATATTGAAGGAGAAAGCAGGTCTG-3′	297bp
*KIF*1.2	F:5′-CACTTGTGGAAAGCCCATTGG-3′ R:5′-AAGGCTGGTCCCAGAAGTGGAACT-3′	158bp
*Kap*2.12	F:5′-ACCGACCGTCTGCTGAAAAAGTT-3′ R:5′-GATACAAGAAGGGAAGAAGGAA-3′	166bp
*Kap*4.2	F:5′-CTCCCTTTTTCTTCAGCACATC-3′ R:5′-GCCAGTAGGAGGACATTTATTT-3′	168bp
18S rRNA	F:5′-CGGTCGGCGTCCCCCAAC-3′ R:5′-GCGTGCAGCCCCGGACATCTAA-3′	103bp

gene of Ct test sample - internal reference gene of Ct test sample) - (target gene of Ct calibration sample - internal reference gene of Ct calibration sample).

3. Results

3.1. Structure of Hetian sheep wool follicle at different concentrations

Skin samples were analyzed on the fourth day of androgen treatment for samples treated with androgen concentrations of 10 nM, 100 nM, 1000 nM and 0 nM (control). SACPIC dyeing of wool follicles (Figure 1) showed that treatment of wool follicles with an androgen concentration of 1000 nM resulted in severe atrophy of both wool follicle outer root sheath and wool bulb structures as compared to corresponding structures of untreated control follicles. Meanwhile, the 10 nM androgen treatment group and control group exhibited no significant difference, possibly because an androgen concentration of 10 nM was too low to affect the wool follicle. At a concentration of 1000 nM, the hairy nucleus of the dermal papilla is deeply stained, the hair shaft is slender, and the hair follicle bulge is incapable of appearing. This is a typical structural change of the hair follicle into the degenerative phase. However, in the 100 nM androgen-treated group, proliferation was observed beneath the outer root sheath and among wool bulb dermal papillary cells.

3.2. Statistical analysis of wool follicle length

According to Figure 2, rapid growth of untreated mountain type Hetian sheep wool follicles was observed during the first three days of culture that then slowed by the fourth day and remained slow thereafter. The growth rate of wool follicles treated with an androgen concentration of 100 nM was higher than that of the control group on average. Conversely, the growth rate of wool follicles cultured at an androgen concentration of 1000 nM was lower than that of the control group, while the growth rate of wool follicles cultured at an androgen concentration of 10 nM was almost equal to that of the control group. The *P*-values (Table 2) for results for each concentration showed that only the difference between mean growth length of specimens treated with 100 nM androgen and the control group was statistically significant ($P = 0.001$).

According to the statistics shown in Figure 3, the growth rate of untreated plains variety Hetian sheep wool follicles was high during the first three days of culture then slowed by the fourth day or androgen-treated specimens. *P*-values (Table 3) for results for different androgen concentrations showed that the difference in average growth length between the 100 nM androgen treatment group versus the control group was statistically significant ($P=0.009$). No significant differences between wool follicle growth length for cultures containing other

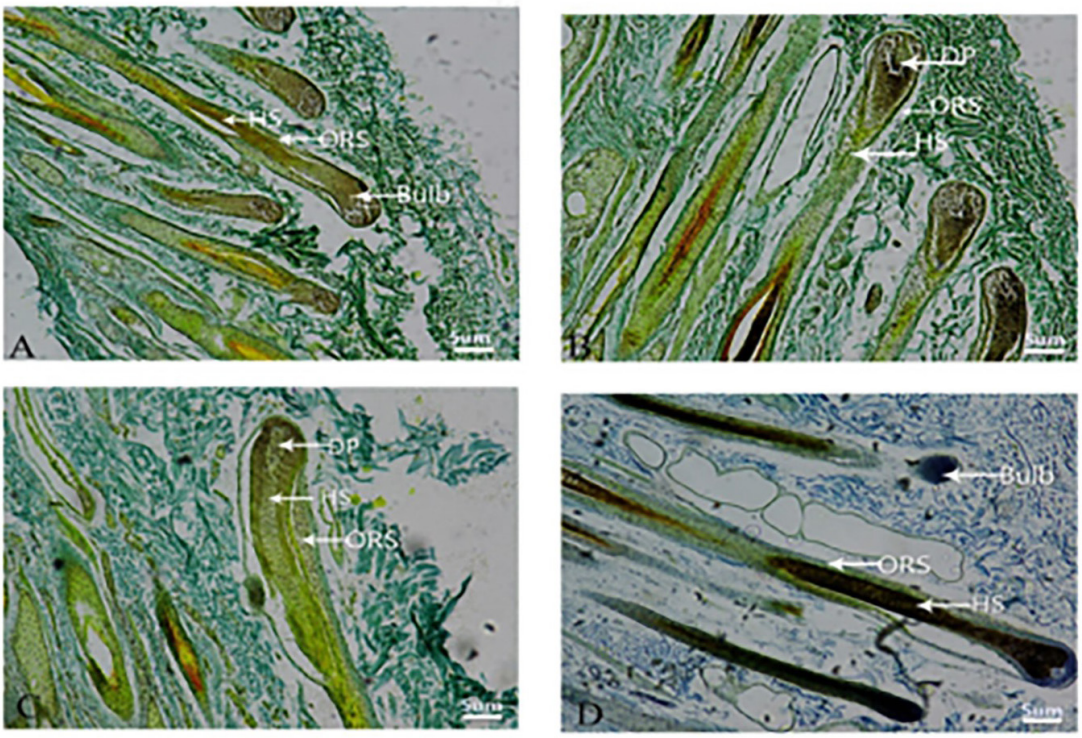

Figure 1. SACPIC staining of Hetian sheep wool follicle (×100). (A) Cultured for four days at 0 nmol concentration; (B) Cultured for four days at 10 nmol concentration; (C) Cultured for four days at 100 nmol concentration; (D) Cultured for four days at 1000 nmol concentration; Note: HS: Hair shaft; ORS: Outer root sheath. DP: Dermal papillae; Bulb: follicle bulb.

androgen concentrations versus control were observed for skin samples from mountain variety Hetian sheep.

3.3. Proliferation markers EdU staining of Hetian sheep wool follicle

Acorrding to Figure 4, on various days of control sample incubation without androgen, bursa cell proliferation markers were observed on the second day of culture that were mainly concentrated within wool bulb cells of dermal papillae. By the fourth day, extending roughly to the wool bulb above the cell migration zone, dermal papillary structures were visible and a small number of wool papilla were observed. On the sixth day, proliferating cells were largely absent, with a distribution of scattered cell proliferation observed in areas such as the wool follicle outer root sheath, where the wool follicles appeared to adopt a resting phase growth pattern.

For Hetian sheep wool follicles treated with 100 nM androgen and cultured for various numbers of days (Figure 5). The experimental group showed significantly higher cell proliferation expression than the control group at 2 days of EdU labeling, and mainly concentrated in the dermal papilla region. By the fourth day, dermal papillary cells site gradually weakened on the fourth day, and the labeled proliferating cells gradually migrated

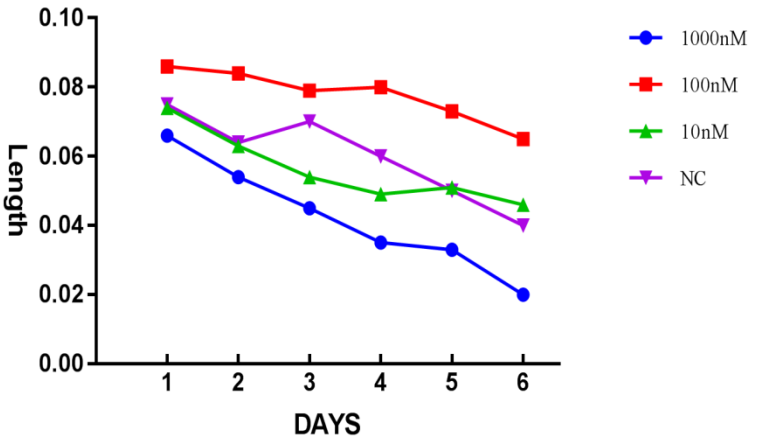

Figure 2. Average daily growth length of mountain type Hetian sheep wool follicle Note: NC:Negative control.

Table 2. Average daily growth length of mountain type Hetian sheep wool follicle.

CONC	Daily average length (mm)	Total average length (mm)	*P*-values
10 nmol	0.056	0.34	*P*=0.32
100 nmol	0.079	0.48	*P*=0.001
1000 nmol	0.042	0.27	*P*=0.02

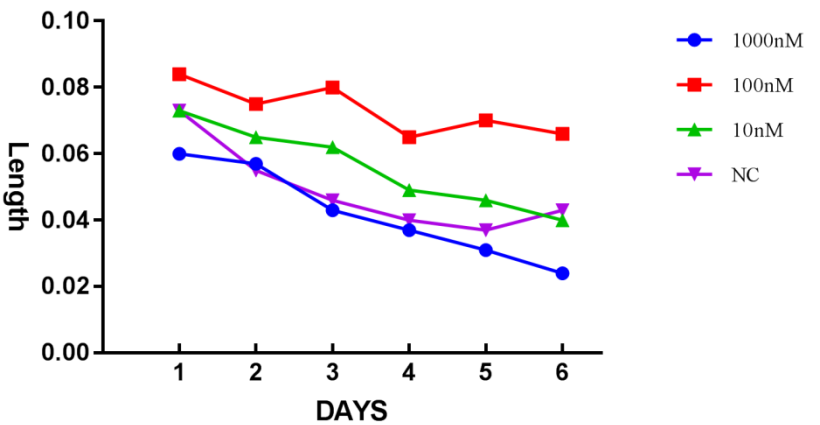

Figure 3. Statistics of average daily growth length of plain type Hetian sheep wool follicle. Note: NC:Negative control.

Table 3. Average daily growth length of plain type Hetian sheep wool follicle.

CONC	Daily average length (mm)	Total average length (mm)	*P*-values
10 nmol	0.055	0.34	*P*=0.063
100 nmol	0.073	0.44	*P*=0.009
1000 nmol	0.039	0.25	*P*=0.075

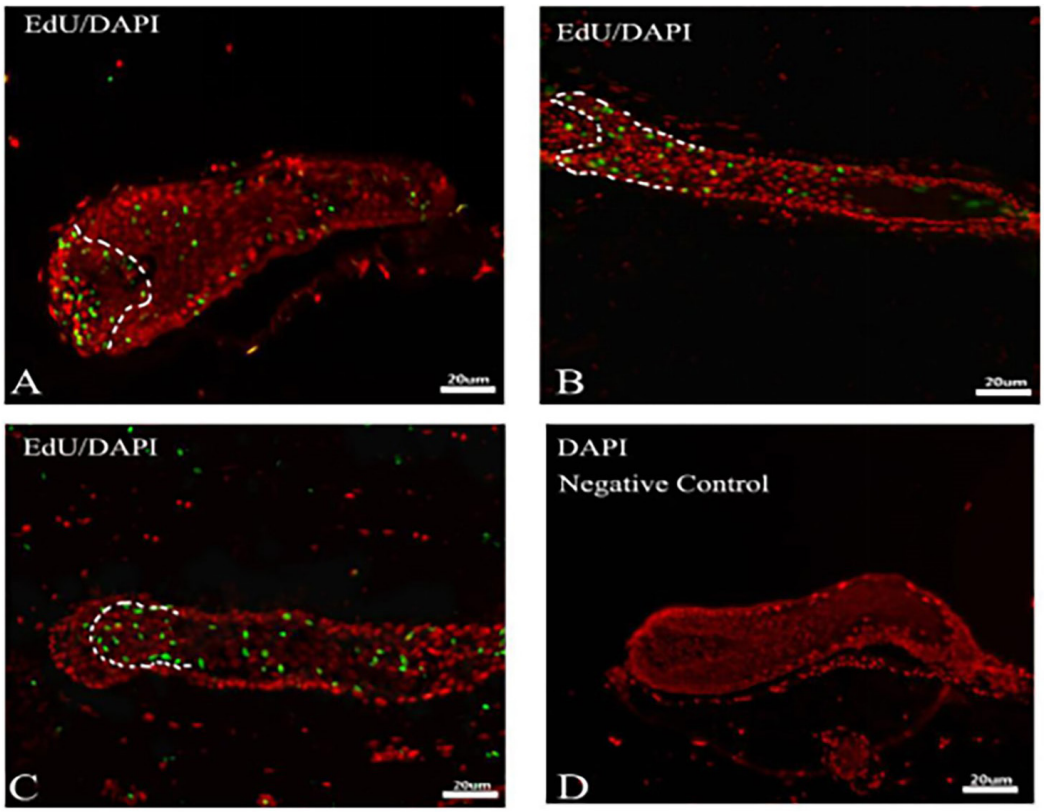

Figure 4. Marker areas of active proliferation of Hetian sheep wool follicle cells in control group at different culture times. (A) Without DHT culture for 2 days; (B) Without DHT cultured for 4 days; (C) Without DHT cultured for 6 days; (D) PBS was used to replace the negative control of secondary antibody. Note: The green fluorescence was EdU labeled proliferating cells, and the red fluorescence was the nucleus.

to the outer root sheath of the wool follicle and the vicinity of the hair shaft. On the 6th day of culture, cell proliferation activity appeared to have weakened and cells tended to accumulate in the root sheath and hair shaft of androgen-treated wool follicles as compared with the control group. However, at day 6 a small amount of cell proliferation was still observed to occur in wool bulbs of the experimental group.

3.4. TUNEL apoptosis staining of Hetian sheep wool follicle

Images (Figure 6) of the control group without androgen show that marked and scattered apoptosis was visible in wool follicle cells at 1 d of culture. By 4 d of culture, apoptotic cells increased gradually that was concentrated within the wool follicle outer root sheath and wool bulb. At day 6 of culture, in control samples without androgen apoptosis was apparent throughout the wool follicle, with visible contraction of wool follicles and fracture of outer root sheaths likely resulting from apoptosis. In the presence of androgen, marked apoptosis was also observed by culture day 6, but with delayed wool follicle development observed *in vitro*.

According to the apoptosis of Hetian wool sacs in different days of androgen culture (Figure 7), it was found that the cells were scattered and the apoptotic cells were scattered on the second day of culture. As the culture time prolonged, the number of apoptotic cells also increased. The increasing and occurring parts are also obvious, and there is more apoptosis in the wool bulbs. It may be that the hair bulbs belong to the germinal center of the cells, and the cells are more proliferated. However, the cells rarely appear in the hairy head. Apoptosis indicates that stem cells in the dermal papilla are still dividing and growing. By the sixth day, apoptotic cells were mainly concentrated in the periphery of the hair bulb. The apoptotic cells in the

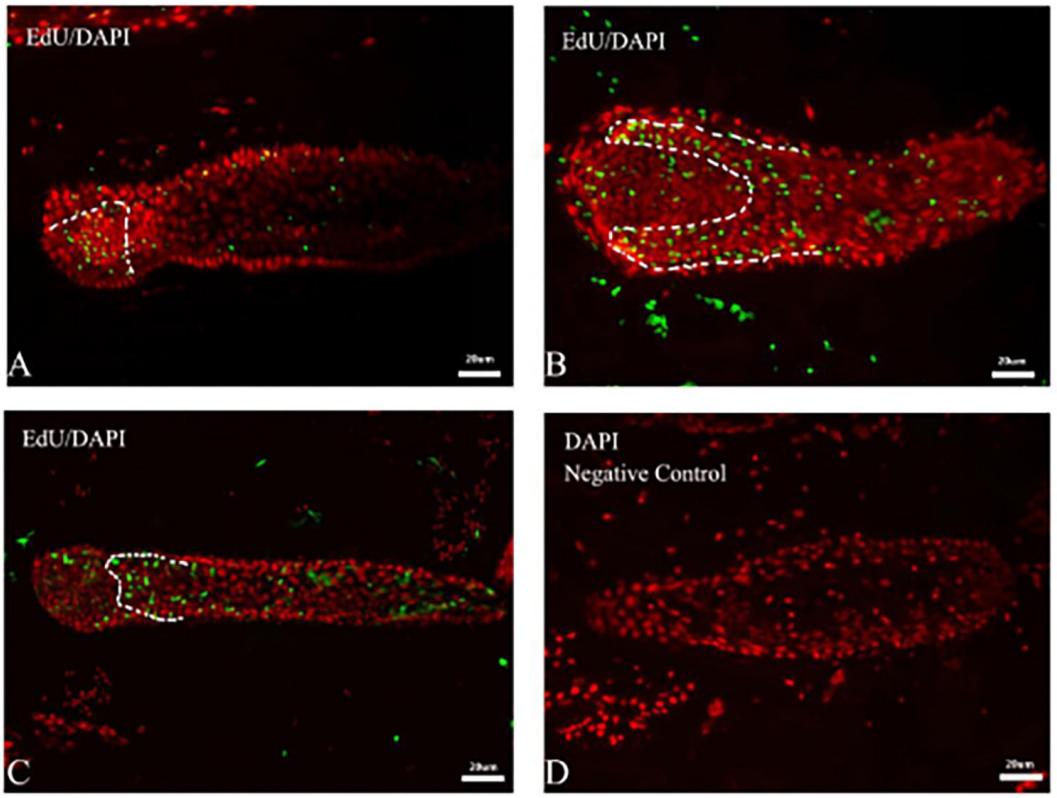

Figure 5. Markers of active cell proliferation in Hetian sheep wool follicle cells of the experimental group at different culture times. (A) DHT was added for culture for 2 days; (B) DHT was added for culture for 4 days; (C) DHT was added for culture for 6 days; (D) PBS replaced the negative control with secondary antibody. Note: The green fluorescence was EdU labeled proliferating cells, and the red fluorescence was the nucleus.

center of the hair bulb and the outer root sheath of the hair follicle were significantly less, probably because androgen delayed the apoptosis of the cells.

3.5. Quantitative Real-time PCR analysis of Hetian sheep wool follicle

Quantitative real-time PCR analysis was conducted for all genes mentioned below using the same method for each, whereby gene expression levels of androgen-treated wool follicle of mountain and plains Hetian sheep were compared to expression levels of the same genes for the untreated control group (using triplicate samples). Expression levels were normalized to levels for an internal 18S ribosomal DNA gene using the $2^{-\Delta\Delta Ct}$ method. Normalized values were then used to calculate mean standard deviations of triplicate gene expression level data. Finally, differences in gene expression level between groups were analyzed for significance via T-test analysis. Quantitative PCR results (Figure 8) here showed that *Kap1.1, KIF1.2, Kap2.12* and *Kap4.2* gene expression level of the mountainous experimental group was significantly higher than plains Hetian sheep. For the keratin high sulfur protein family gene *Kap1.1* that controls wool thickness. Wool follicle keratin intermediate filaments protein gene *KIF1.2*, encodes a protein that has been shown to control wool glossiness. The high sulfur protein family member gene *Kap2.12* encodes a basic protein and the *Kap4.2* gene codes for a slightly alkaline protein that belongs to the ultra-high sulfur protein family.

4. Discussion

The growth cycle of hair follicles is generally divided into three stages: growth phase, regression and resting period. The growth cycle of hair follicles is regulated by a variety of cytokines and hormones, including androgen (He et al., 2017). Studies have shown that the Wnt/β-catenin pathway plays an important role in inducing hair follicle production and promoting hair follicle growth, with androgen playing an important role (Lu et al., 2004). According to previous studies, expression levels of Wnt3a, β-catenin, VEGF and Wnt10b, all of which promote hair growth, differ under the action of androgen (Olsson et al., 2006). In this study, the optimal concentration range of androgen was determined via *in vitro* culture of Hetian sheep wool follicles to assess androgen effects on Kap gene expression, as determined via immunodetection of follicle cell proliferation and apoptosis.

Wool follicles were isolated by microscopic dissection, which preserved the activity and integrity of follicles.

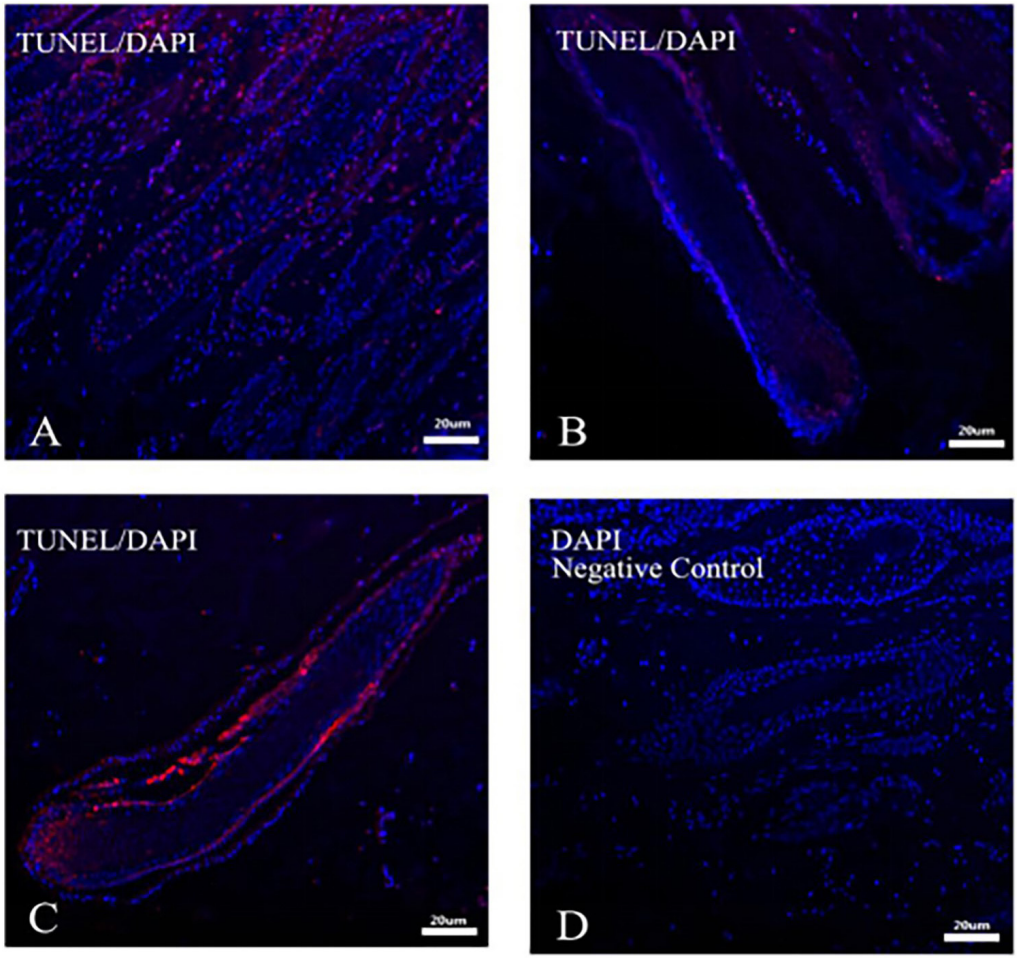

Figure 6. Apoptotic marker areas of wool follicle cells in the control group Hetian sheep at different culture times. (A) Without DHT culture for 2 days; (B) Without DHT culture for 4 days; (C) Without DHT culture for 6 days; (D) PBS was used to replace the negative control of secondary antibody. Note: The red fluorescence was TUNEL-labeled apoptotic cells, and the blue fluorescence was the nucleus

Using four androgen concentration gradients, the growth state of wool follicles during the first 4 days of culture with 100 nM androgen was shown to exceed that of the control group, with a statistical significance of $P< 0.05$. However, after day 4, the growth state of wool follicles slowed. The underlying reason for this observation warrants further study, but a preliminary explanation may be related to hair follicle cycle regression and rest stages and the conditions used here for *in vitro* culture.

Immunofluorescence staining was next used to investigate the effects of androgen on cell proliferation levels within hair follicles. At present, many markers are available for use as cell proliferation markers. Among them, EdU markers have low toxicity and little effect on cell growth, as demonstrated previously (Amoh et al., 2005; Hu et al., 2004; Huntriss et al., 2006; Wu et al., 2009; Kerrigan et al., 1997) in apoptosis labeling assays that utilize *in situ* end labeling to detect DNA cleavage, including an abbreviated TUNEL method (Jaana and Suvi, 2020). Here it was observed that androgens had a great effect on proliferation of cells within the hair bulb region, with fewer apoptotic cells observed during the period of cell proliferation and more apoptotic cells observed during hair follicle regression period. Comparison between hair follicles in the androgen-free group and those in the androgen-treated group showed that androgens could delay and reduce apoptosis duration and numbers of apoptotic cells, while promoting proliferation of stem cells in the dermal papilla of the bulb. In addition, these results also indirectly indicate that an appropriate concentration of androgen can promote hair follicle growth.

It has been experimentally demonstrated that androgen has a great effect on cell proliferation within the hair bulb (Brancaz et al., 2004). Few apoptotic cells are present during the cell proliferation period, but more apoptosis occurs after hair follicles enter the regression (degenerative) stage (Aittomäki et al., 1995). During this stage, as compared to the non-androgen group, development of

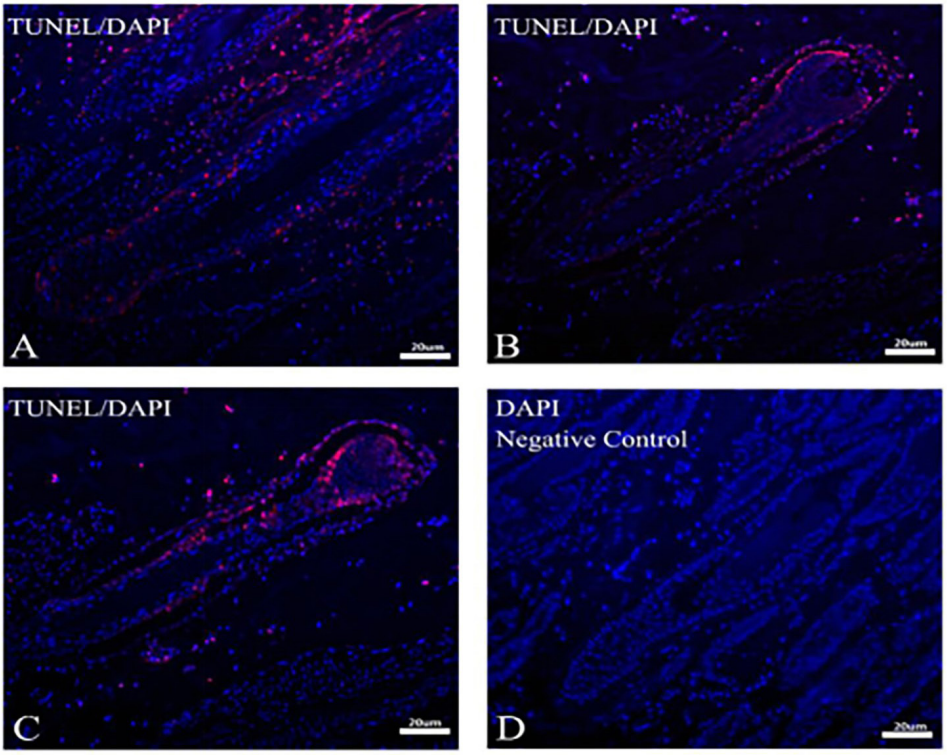

Figure 7. Apoptotic marker region of wool follicle cells in the experimental group Hetian sheep at different culture time. (A) DHT was added for culture for 2 days; (B) DHT was added for culture for 4 days; (C) DHT was added for culture for 6 days; (D) PBS was used to replace the negative control of secondary antibody. Note: The red fluorescence was TUNEL-labeled apoptotic cells, and the blue fluorescence was the nucleus.

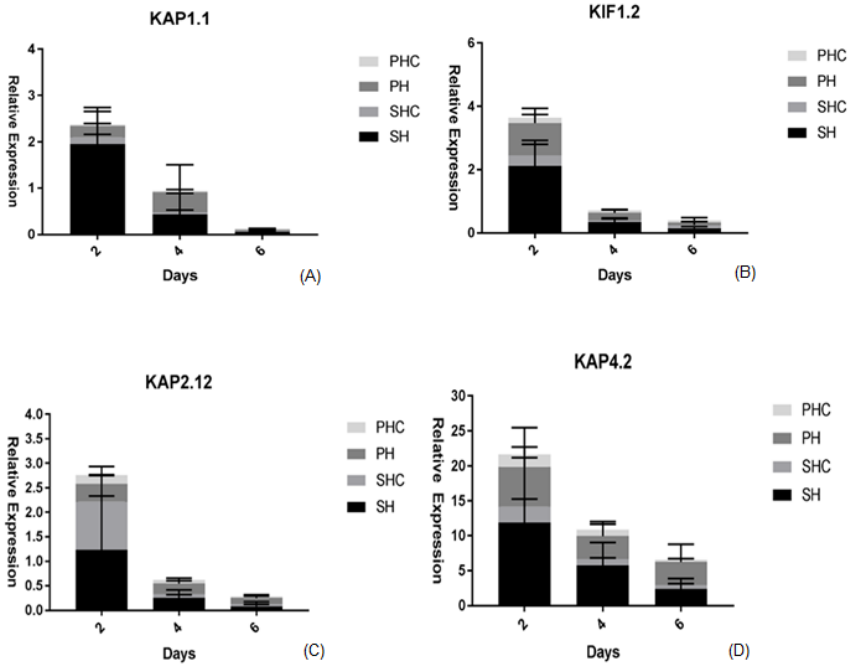

Figure 8. Significance chart of the difference of *Kap* and *KIF* genes between two type of Hetian sheep. *Kap* 1.1 Relative expression (A); *KIF* 1.2 Relative expression (B); *Kap* 2.12 Relative expression (C); *Kap* 4.2 Relative expression (D). Note: PHC: plain type sheep control group. PH: plain type sheep experimental group. SHC: mountain type sheep control group. SH: mountain type sheep experimental group.

androgen-treated hair follicles is delayed, with reduction in time and quantity of cell apoptosis and promotion of proliferation of stem cells observed within dermal papillae of hair bulbs. Notably, this result also indirectly indicates that an appropriate concentration of androgen can promote growth of hair follicles.

It has been shown that the general quality of semi-coarse wool in Xinjiang Hetian mountain sheep is superior to that of plains sheep with regard to wool luster and yield. Therefore, hair follicle responses to androgen or no androgen treatments of both Hetian sheep varieties were compared that androgen-treated wool follicles significantly differed from that of untreated follicles. Quantitative real-time PCR analysis showed that *Kap* genes expression was relatively high during the first four days of androgen stimulation, with differences observed in response levels of various *Kap* genes to androgen. Expression levels of *Kap1.1*, *KIF1.2*, *Kap2.12*, *Kap4.2*. Notably, among *Kap* genes, some genes of mountain variety and plains variety Hetian sheep that code for intermediate silk protein and super high sulfur proteins differed in their expression patterns both with and without added androgen. Such effects may explain differences in wool characteristics between the two Hetian sheep varieties, which exhibit differences in wool hair shaft diameter and gloss. These results therefore lay a theoretical foundation upon which to improve the quality of Hetian sheep wool in the future.

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