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Original Research Article

Pyridoxine regulates hair follicle development via the PI3K/Akt, Wnt and Notch signalling pathways in rex rabbits

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ABSTRACT

This study was conducted to evaluate the effect of pyridoxine on the development of hair follicles in Rex rabbits and the underlying molecular mechanism. Two hundred 3-month-old Rex rabbits were randomly divided into 5 groups and fed diets supplemented with 0, 5, 10, 20, or 40 mg/kg pyridoxine. The hair follicle density on the dorsal skin and the gene and protein expression levels of components of the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or Akt), Wnt, Notch and bone morphogenetic protein (BMP) signalling pathways were measured. In addition, free hair follicles were isolated from Rex rabbits and cultured with pyridoxine in vitro to measure hair shaft growth. Furthermore, dermal papilla cells (DPC) were isolated from the skin of Rex rabbits and cultured with pyridoxine in vitro to measure the gene and protein expression levels of components of the PI3K/Akt, Wnt, Notch and BMP signalling pathways. The results showed that the addition of dietary pyridoxine significantly increased the total follicle density, secondary follicle density, and secondary-to-primary ratio (S/P, $P < 0.05$), that the growth ratio of hair stems was promoted by pyridoxine in basic culture medium, and that the growth length of tentacle hair follicles cultured in the pyridoxine group was longer than that in the control group ($P < 0.05$). In addition, pyridoxine changed the DPC cycle progression and promoted cell proliferation, and appropriate concentrations of pyridoxine (10 and 20 μmol/L) significantly inhibited cell apoptosis ($P < 0.05$). Pyridoxine significantly affected the gene expression of components of the PI3K/Akt, Wnt and Notch signalling pathways in the skin and DPC of Rex rabbits ($P < 0.05$), increased the levels of phosphorylated catenin beta 1 (CTNNB1) and Akt, and decreased the level of phosphorylated glycogen synthase kinase 3 beta (GSK-3β) ($P < 0.05$). Therefore, the molecular mechanism by which pyridoxine promotes hair follicle density in Rex rabbits probably occurs through activation of the PI3K/Akt, Wnt and Notch signalling pathways, prolonging hair follicle growth and delaying the onset of telogen.

1. Introduction

Hair follicles are part of the skin epithelium in vertebrates and are characterized by periodic growth during adult life (Millar, 2002). Many different cells and signalling pathways interact with each other to regulate hair follicle development. Dermal papilla cells (DPC), hair matrix cells, inner root sheath cells, and outer root sheath cells are important cellular components of hair follicles and participate in hair follicle development (Fuchs, 2007; Driskell et al., 2011; Chi et al., 2013). Moreover, many studies have reported that

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different signalling pathways, such as the Wnt, Notch, bone morphogenetic protein (BMP), and fibroblast growth factor (FGF) pathways, participate in hair follicle development (Kulessa et al., 2000; Foitzik et al., 2000; Su et al., 2008; Demehri and Kopan, 2009; Lin et al., 2015; Zhang et al., 2016). The Notch signalling pathway interacts with the BMP signalling pathway, which can inhibit the Wnt signalling pathway by regulating β-catenin (Demehri and Kopan, 2009). Signal exchange between hair follicle stem cells and DPC is the key to initiating the next hair follicle cycle (Wu et al., 2010; Woo et al., 2013).

Vitamins are trace organic resources that must be obtained from food to maintain normal physiological function, and they play important roles in animal growth, metabolism and development. Vitamin B6 is a general term for pyridine compounds, including pyridoxine, pyridoxal, pyridoxine and their phosphate forms; it was discovered almost one century ago (György, 1956; Hellmann and Mooney, 2010; Kraemer et al., 2012; Eggersdorfer et al., 2012), and it is the active form of pyridoxal-5′-phosphate (PLP), which serves as a cofactor for a wide variety of proteins and enzymes (Jansonius, 1998; Christen and Mehta, 2001; Eliot and Kirsch, 2004; Phillips, 2015). According to reports, PLP functions as a cofactor of more than 160 enzymes involved in the biosynthesis of amino acids (John, 1995; Eliot and Kirsch, 2004; Percudani and Peracchi, 2009). The formation of fur on animal skin requires a large amount of protein and sulfur-containing amino acids, but there are few studies on the molecular mechanism by which pyridoxine regulates hair follicle morphogenesis in Rex rabbits. In other words, the molecular mechanism by which pyridoxine participates in regulating hair follicle development is not clear. The purpose of this study was to investigate the effect of pyridoxine on hair follicle development in vivo and in vitro, and the molecular mechanism was preliminarily discussed.

2. Materials and methods

2.1. Animals, free hair follicles, DPC and experimental design

2.1.1. Animals, feeding and experimental design in feeding

Two hundred 3-month-old Rex rabbits with similar body weights were randomly assigned to 1 of 5 diets, with 40 rabbits in each experimental diet group. The experimental diet formula composition and nutritional level met the recommended nutrient requirements for juvenile rabbits (NRC 1977, Table 1), and the following 5 concentrations of pyridoxine were added to the experimental diets: 0, 5, 10, 20 and 40 mg/kg (98% pyridoxine hydrochloride, Jiangxi Tyson Pharmaceutical Co., Ltd., China; as-fed basis). At the end of the 60-d feeding trial (53-d experimental period and a 7-d adjustment period), 8 rabbits per dietary group (4 males and 4 females) with body weights close to that of the average body weight of the group were bled by cardiac puncture. After shearing, the middle part of the back skin were collected with cryopreservation tube, frozen in liquid nitrogen, and stored at −80 °C for mRNA and protein expression analysis.

2.1.2. Free hair follicle culture in vitro and experimental design in organ

The whisker hair follicles from 30-d-old Rex rabbits were used in this study to establish organ cultures. Small skin pieces (1 cm^2) of whisker were excised from Rex rabbits as previously described in mice (Ouji et al., 2007). Tentacle hair follicles were divided into 6 groups, with 10 follicles in each group. The extracted hair follicles were placed in 24-well plates. The concentrations of pyridoxine (Solarbio, China) in basic culture medium were 0, 10, 20, 40, 80 and 160 μmol/L, and suspended culture was carried out at 31 °C and 5% CO_2. The basic culture medium composition was Williams E medium (Sigma–Aldrich, USA), 2 mmol/L L-glutamine (Sigma–Aldrich, USA), 10 μg/mL insulin (Sigma–Aldrich, USA), 10 ng/mL hydrocortisone (Sigma–Aldrich, USA), 100 U/mL

Table 1
Composition and nutrient levels of the experimental diet (%, dry matter basis).

Ingredients	Content	Nutrient levels[2]	Content
Corn	15.0	Digestible energy, MJ/kg	11.42
Barley grain	10.0	Crude protein	18.00
Wheat bran	12.0	Ether extract	3.10
Soybean meal	10.0	Crude fibre	19.41
Sunflower meal	8.0	Crude ash	13.06
Peanut vine straws	30.0	Calcium	1.08
Rice hull powder	10.0	Phosphorus	0.48
Premix[1]	5.0	Lysine	0.67
Total	100.0	Methionine	0.30

[1] The premix provided the following per kilogram of diet, vitamin A: 10,000 IU; vitamin D_3: 2,000 IU; vitamin E: 50 mg; vitamin K_3: 2.5 mg; choline chloride: 400 mg; thiamine: 5 mg; riboflavin: 10 mg; nicotinic acid: 20 mg, pantothenic acid: 50 mg; folic acid: 2.5 mg; cobalamin: 1 mg; Fe: 100 mg; Zn: 50 mg; Cu: 40 mg; Mn: 30 mg; I: 0.5 mg; Se: 0.05 mg; calcium hydrogen phosphate: 1.5 g; sodium chloride: 5 g; lysine: 1.5 g; methionine: 1.5 mg; the rest is miscellaneous meal carrier complement.

[2] Digestible energy was calculated according to "Tables of feed composition and nutritive values in China (The 20th revised edition, 2009), Institute of animal science of CAAS (Chinese Academy of Agricultural Sciences, 2019)", while the others were measured values.

Table 2
Information of primers.

Gene	Accession number	Primer sequence (5′-3′)	Product length, bp
Inppl1	NM_017349797	F: CAGAGCACGAGAACCGCATCAG R: AGCCGCAGGATGTCCAGGTAG	199
Inpp4b	NM_008267416	F: TCCTAAGAGCACAGCGGAGAGC R: GCTGCCTTCACTGCCACCATC	195
Frk	XM_008263392	F: CAAGCGATGGCCTCTGTGTCAG R: CTGCTACTGGAGTGGTGTTGTTCC	195
Phlda3	XM_002717603	F: GCGGCGGCGAGATTGACTTC R: CTGGATGGCCTGCTGGTTCTTG	101
Wnt10b	NM_002711076	F: TGTGCCATCCCTCTTCCTTA R: GGCTCCACCTCTAACTTCTGC	150
CTNNB1	DQ786777	F: TTCTTGGGACTCTTGTTCAGC R: CACTTGGCACACCATCATCT	122
GSK-3β	NM_001146156	F: ATCCATGTCTCCCTGTCCAC R: TTTCCTCTTCCCACTCCTGA	119
DKK1	NM_001082737	F: ATTCCAACGCCATCAAGAAC R: CCACACTCCTCGTCCTCTGT	163
Notch1	XM_011518717	F: TGCGAGACCAACATCAACGAGTG R: TCAGGCAGAAGCAGAGGTAGGC	94
JAG1	XM_018261778	F: TGGAGGAGGACGACATGGACAAG R: CATCCGATTGAGGCTCTGTGCTG	176
Hes1	XM_002716517	F: CCAGATCAACGCCATGACCTATCC R: ACACCTTAGCCGCCTCTCCAG	200
Hes5	NM_008253710	F: AGACCGCATCAACAGCAGCATC R: ATCTCCAGGATGTCCGCCTTCTC	105
BMP2	XM_001082650	F: GACATCCTGAGCGAGTTCGAGTTG R: CGGCGGTACAAGTCCAGCATG	113
BMP4	NM_001195723	F: CTAAGCATCACCCACAGCGG R: CAGTCATTCCAGCCCACGTC	163
TGF-β1	NM_008249704	F: 5′- CCGTTTCTTTCGTGGGATAC R: GGTAAGGGAGGAGGGTCTCA	108
GAPDH	NM_001082253	F: TGCCACCCACTCCTCTACCTTCG R: CGAAGGTAGGGATGGGTGGCA	118

Inppl1 = inositol polyphosphate phosphatase like 1; F = forward primer; R = reverse primer; *Frk* = fyn-related kinase; *Phlda3* = pleckstrin homology like domain family A member 3; *CTNNB1* = catenin beta 1; *GSK-3β* = glycogen synthase kinase 3 beta; *DKK1* = dickkopf-1; *JAG1* = Jagged1; *BMP* = bone morphogenetic protein; *TGF-β1* = transforming growth factor-β; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

Table 3
Effects of pyridoxine on dry matter intakes and hair follicle density of Rex rabbit[1].

Item	Pyridoxine level, mg/kg					RMSE	*P*-value
	0	5	10	20	40		
Dry matter intakes, g/d	128.47^{c}	129.24^{bc}	131.81^{b}	134.58^{a}	130.50^{bc}	31.301	0.001
Total hair follicle density, count/mm^2	258.83^{b}	286.22^{b}	318.50^{ab}	395.71^{a}	338.92^{b}	64.795	0.002
Primary hair follicle density, count/mm^2	14.77	17.00	15.23	14.38	14.90	3.513	0.604
Secondary hair follicle density, count/mm^2	244.07^{c}	269.21^{bc}	302.98^{bc}	381.33^{a}	324.02^{ab}	65.240	0.002
Secondary-to-primary ratio (S/P)	17.13^{b}	16.48^{b}	21.42^{ab}	28.63^{a}	22.54^{ab}	8.363	0.044

$^{a, b, c}$ Within a row, means with different superscript letters are significantly different ($P < 0.05$).
[1] Data represent means and root mean error (RMSE), $n = 40$ for dry matter intakes, and $n = 8$ for hair follicle density.

penicillin (Solarbio, China), and 100 μg/mL streptomycin (Solarbio, China). The medium was changed every 2 d. The growth length of the fur wool stem was measured every 24 h from the beginning of follicle culture (0 to 144 h).

2.1.3. Isolation and culture of DPC and experimental design in cell

DPC were isolated from median skin of back pieces from 30-d-old Rex rabbits and cultured according to previously reported methods (Liu et al., 2020a). Third-generation DPC were inoculated at 10^4 cells/mL in a 6-well plate, with 2 mL of cell suspension per well. After the cells were allowed to adhere for 24 h, the culture medium was removed, and Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific) containing 10% foetal bovine serum (FBS, Gibco, Thermo Fisher Scientific) with pyridoxine (Solarbio, China) concentrations of 0, 10, 20, 40, 80, and 160 μmol/L was added, with 6 replicate wells for each concentration. After 72 h, the cells were collected, and the effects of pyridoxine on the cell proliferation rate, cell cycle progression and apoptosis were evaluated. The gene expression of components of the PI3K/Akt, Wnt, Notch and BMP signalling pathways and the levels of phosphorylated Akt, catenin beta 1 (CTNNB1) and GSK-3β were also detected.

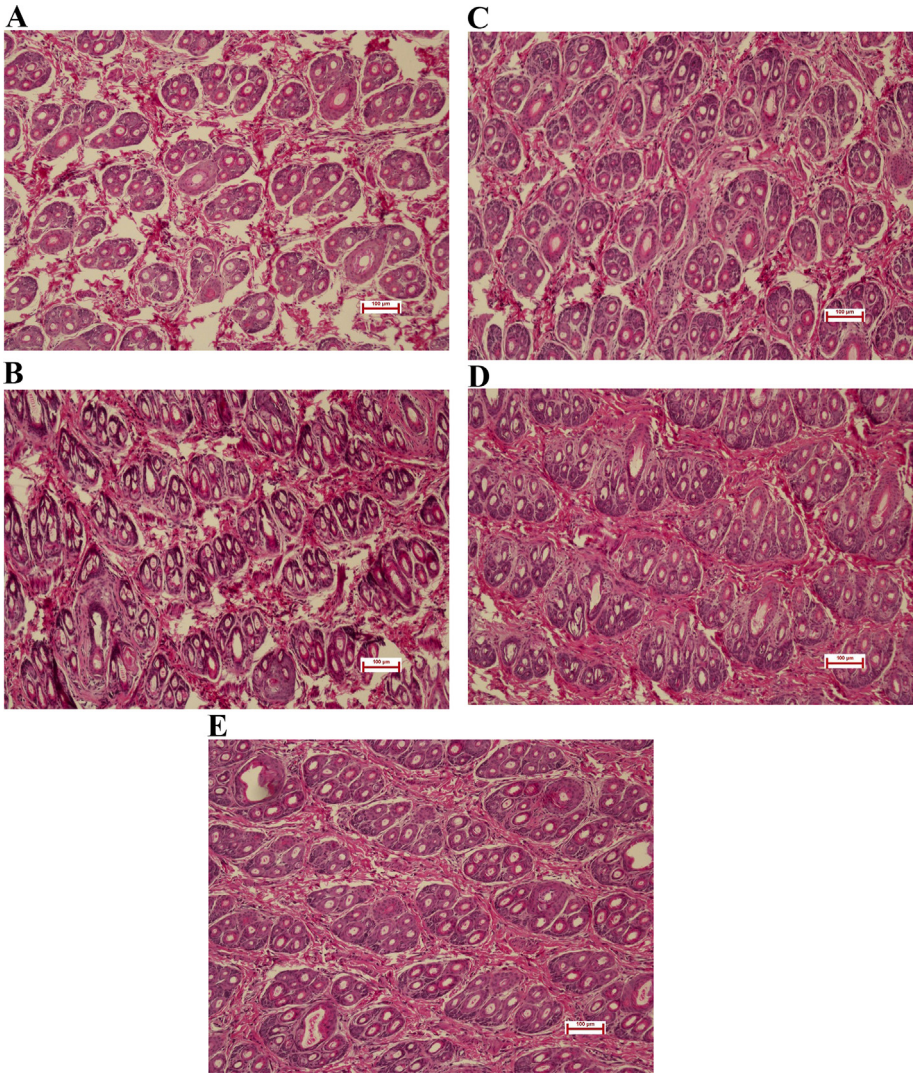

Fig. 1. Effects of dietary pyridoxine supplemented level on hair follicle density of Rex rabbit. (A) Pyridoxine were supplemented 0 mg/kg; (B) pyridoxine were supplemented 5 mg/kg; (C) pyridoxine were supplemented 10 mg/kg; (D) pyridoxine were supplemented 20 mg/kg; (E) pyridoxine were supplemented 40 mg/kg. Scale bars = 100 μm.

Table 4
Effects of pyridoxine on gene expression of signal pathway of Rex rabbit[1].

Gene	Pyridoxine level, mg/kg					RMSE	*P*-value
	0	5	10	20	40		
PI3K/Akt signal pathway							
Inppl1	1.00^{b}	0.96^{b}	1.59^{ab}	2.00^{a}	1.47^{ab}	0.541	0.014
Inpp4b	1.00	1.17	1.26	1.29	1.14	0.270	0.391
Frk	1.00	1.24	1.35	1.33	1.33	0.283	0.201
Phlda3	1.00	0.94	1.09	1.15	1.02	0.346	0.896
Wnt signal pathway							
Wnt10b	1.00^{b}	0.93^{b}	0.84^{b}	2.73^{a}	1.22^{b}	0.951	0.011
CTNNB1	1.00^{b}	0.67^{b}	0.65^{b}	1.63^{a}	1.10^{b}	0.380	0.001
GSK-3β	1.00^{a}	0.76^{ab}	0.74^{ab}	0.43^{bc}	0.40^{c}	0.261	0.003
DKK1	1.00	1.17	1.14	1.06	0.98	0.333	0.804
Notch signal pathway							
Notch1	1.00^{b}	1.07^{b}	1.14^{b}	2.14^{a}	1.20^{b}	0.597	0.016
JAG1	1.00	1.33	1.48	1.97	1.50	0.814	0.374
Hes1	1.00^{c}	1.06^{c}	2.23^{b}	3.36^{a}	1.28^{c}	0.524	<0.001
Hes5	1.00^{b}	1.03^{b}	1.85^{b}	3.73^{a}	2.21^{b}	1.184	0.003
BMP signal pathway							
BMP2	1.00	0.68	0.91	0.93	0.94	0.404	0.697
BMP4	1.00	0.86	0.89	0.83	0.67	0.443	0.788
TGF-β1	1.00	0.92	1.03	1.21	0.93	0.464	0.816

Inppl1 = inositol polyphosphate phosphatase like 1; *Frk* = fyn-related kinase; *Phlda3* = pleckstrin homology like domain family A member 3; *CTNNB1* = catenin beta 1; *GSK-3β* = glycogen synthase kinase 3 beta; *DKK1* = dickkopf-1; *JAG1* = Jagged1; *BMP* = bone morphogenetic protein; *TGF-β1* = transforming growth factor-β.
[a,b,c] Within a row, means with different superscript letters are significantly different ($P < 0.05$).
[1] Data represent means and root mean error (RMSE), $n = 8$ for per group.

2.2. Determinations and analyses

2.2.1. Chemical analysis of the experimental diets

Dry matter (DM, 934.01), crude protein (CP, 954.01), ether extract (EE, 920.39) crude fibre (CF, 932.09) and ash (942.05) were measured according to Association of Official Analytical Chemists ((AOAC International 2005)) procedures. The CP content and EE were determined using a Kjeltec Auto 1030 Analyser and Soxtec 1043, respectively (FOSS Tecator AB, Höganäs, Sweden). The calcium and phosphorus contents were determined by inductively coupled plasma atomic emission spectrometry (ICP-EMS; Optima 8000, PerkinElmer, USA). The lysine and methionine contents in the feed were analysed using an automatic amino acid analyser (Basic L-8900, Japan) as a reference (Liu et al., 2017). The DM intakes for each rabbit were calculated according to total dry matter intakes divided by total experimental days.

2.2.2. Assessment of the proliferation, cell cycle progression and apoptosis of DPC

The effect of pyridoxine on proliferation was measured by thiazolyl blue tetrazolium bromide (MTT; Solarbio, China), and cell cycle progression and apoptosis were measured by PI/RNase standing buffer (BD Biosciences, cat: 550825) and PE Annexin V Apoptosis Detection Kit I (BD Biosciences, cat: 559763), respectively, using flow cytometry (BD Accuri C6, BD). The results were analysed with ModFit LT 5.0 software (Verity Software House, USA).

2.2.3. Total RNA extraction and real-time PCR analysis

Total RNA was extracted from skin tissues or DPC with RNAiso reagent (TaKaRa, Japan) according to a reference (Liu et al., 2017). The PCR primers used in this study (Table 2) were designed using a Premier 5.0 Software and synthesized by Ruibo Biological Engineering Co., Ltd. (Qingdao, China). For quantitative RT-PCR analysis of mRNA, 1 μg of total RNA was used to synthesize cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH Mannheim, Germany). PCR amplification was performed using Fast Start Universal SYBR Green Master Mix (Roche Diagnostics GmbH Mannheim, Germany). The relative mRNA expression levels were calculated using the arithmetic formula $2^{-\triangle\triangle Ct}$ (LiLivak and Schmittgen, 2001).

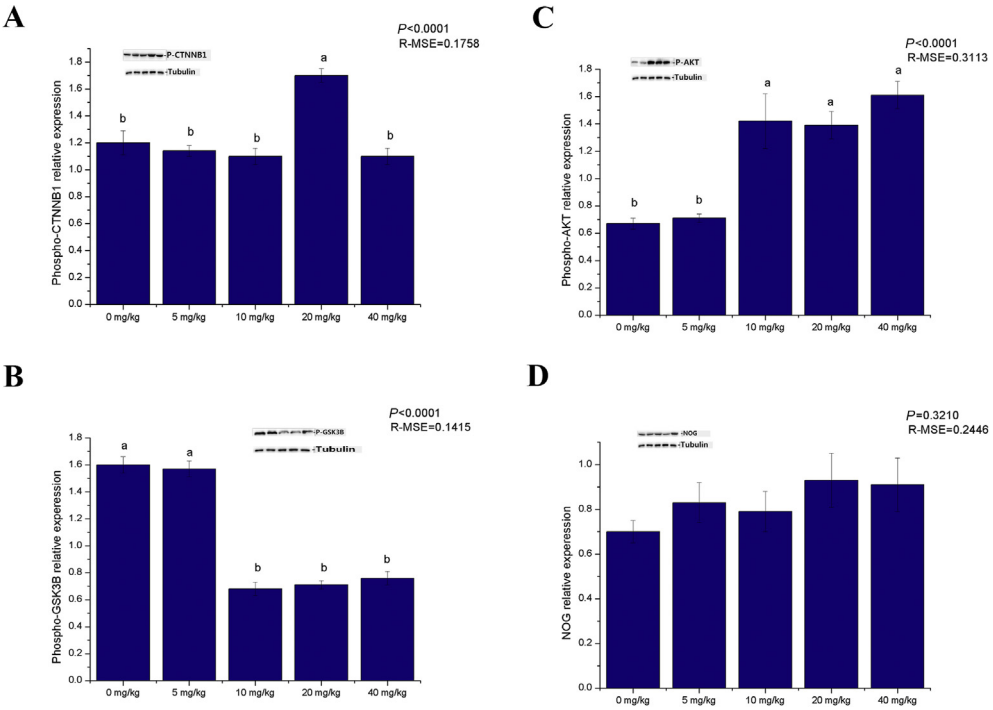

Fig. 2. Effects of pyridoxine on expression of hair follicle-related proteins of Rex rabbit. (A) Phospho-catenin beta 1 (p-CTNNB1); (B) phospho-glycogen synthase kinase 3 beta (p-GSK-3β); (C) phospho-Akt (p-Akt); (D) noggin (NOG) protein. Data represent means ± standard error and root mean error (R-MSE), $n = 8$ for per group. [a, b, c] Bars with different letters means significantly different ($P < 0.05$).

Table 5
Effects of pyridoxine on the growth of free hair follicles in vitro[1].

Item	Pyridoxine concentration, μmol/L						RMSE	*P*-value
	0	10	20	40	80	160		
0 to 24 h, μm	7.20^{b}	7.14^{b}	7.11^{b}	7.30^{b}	9.13^{a}	5.67^{c}	0.693	<0.001
24 to 48 h, μm	5.78^{d}	6.52^{bc}	6.74^{b}	7.19^{b}	9.08^{a}	6.00^{cd}	0.743	<0.001
48 to 72 h, μm	3.38^{c}	2.85^{c}	3.23^{c}	4.69^{a}	5.08^{a}	4.11^{b}	0.625	<0.001
72 to 96 h, μm	2.53^{c}	2.68^{c}	3.08^{c}	3.79^{b}	4.38^{a}	3.02^{c}	0.590	<0.001
96 to 120 h, μm	2.35^{d}	2.71^{cd}	3.15^{bc}	3.89^{a}	4.18^{a}	3.72^{ab}	0.639	<0.001
120 to 144 h, μm	2.10^{c}	2.50^{c}	3.05^{b}	4.03^{a}	4.29^{a}	3.21^{b}	0.517	<0.001
Total length, μm	23.33^{d}	24.39^{cd}	26.37^{c}	30.88^{b}	36.15^{a}	25.72^{c}	2.203	<0.001
Growth ratio, μm/d	3.89^{d}	4.07^{cd}	4.40^{c}	5.15^{b}	6.03^{a}	4.29^{c}	0.367	<0.001

[a, b, c] Within a row, means with different superscript letters are significantly different ($P < 0.05$).
[1] Data represent means and root mean error (RMSE), $n = 10$ for per group.

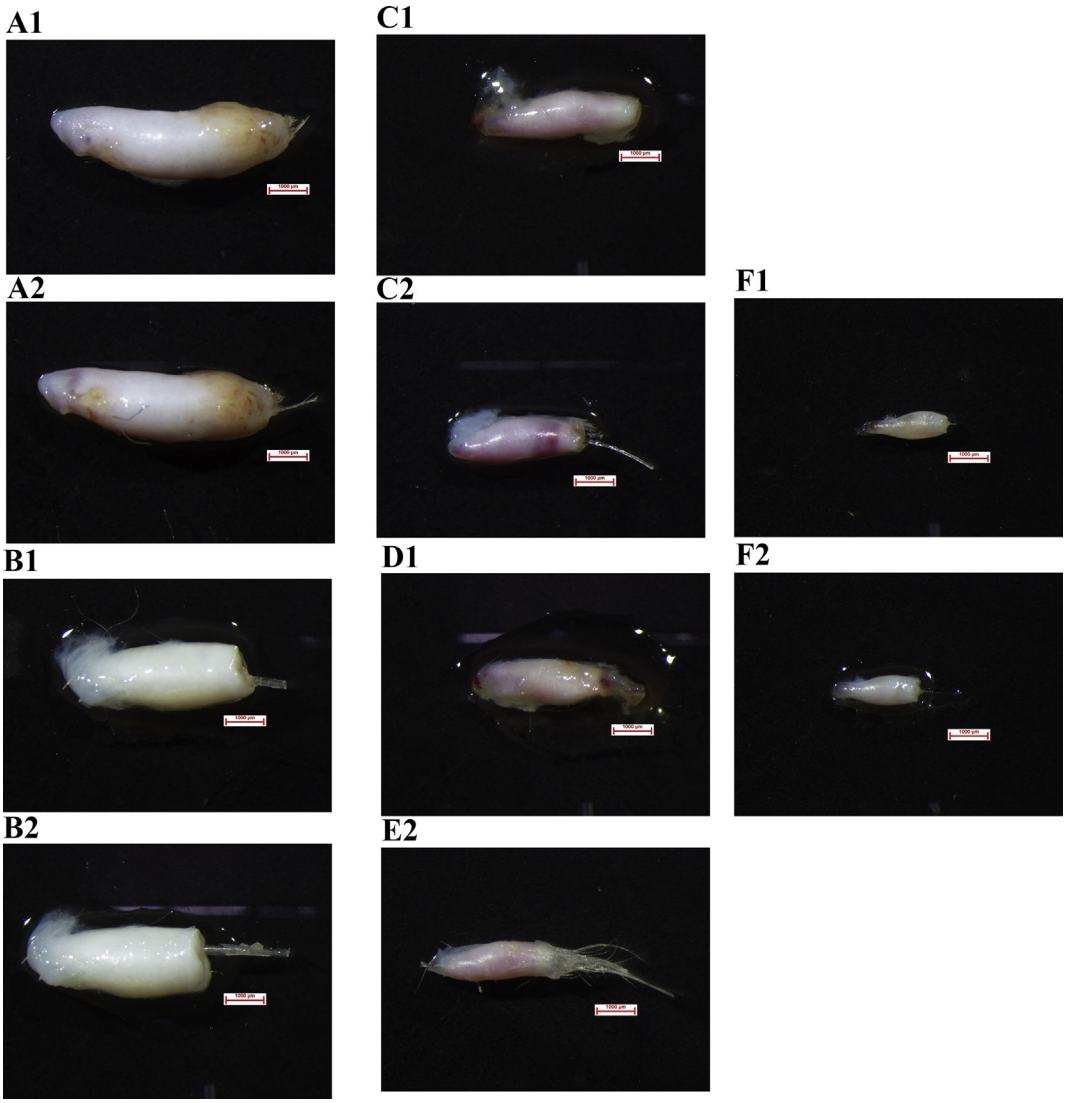

Fig. 3. Effects of pyridoxine on the growth of hair follicles (20× magnification). (A) Pyridoxine were supplemented 0 μmol/L, A1 and A2 represent 0 and 144 h in vitro culture, respectively; (B) pyridoxine were supplemented 10 μmol/L, B1 and B2 represent 0 and 144 h in vitro culture, respectively; (C) pyridoxine were supplemented 20 μmol/L, C1 and C2 represent 0 and 144 h in vitro culture, respectively; (D) pyridoxine were supplemented 40 μmol/L, D1 and D2 represent 0 and 144 h in vitro culture, respectively; (E) pyridoxine were supplemented 80 μmol/L, E1 and E2 represent 0 and 144 h in vitro culture, respectively; (F) pyridoxine were supplemented 160 μmol/L, F1 and F2 represent 0 and 144 h in vitro culture, respectively. Scale bars = 1,000 μm.

2.2.4. Western blotting

Total protein was extracted from skin tissues or DPC using a radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, China), and the protein concentrations were determined using a BCA Protein Assay Kit (Beijing Kangwei Century Biotechnology Co., Ltd., China). The extracted proteins (50 ng/sample) were solubilized in 40 mL of sodium dodecyl sulfate (SDS) loading buffer (Solarbio, China) and then resolved by electrophoresis (Bio-Rad, Richmond, USA) on

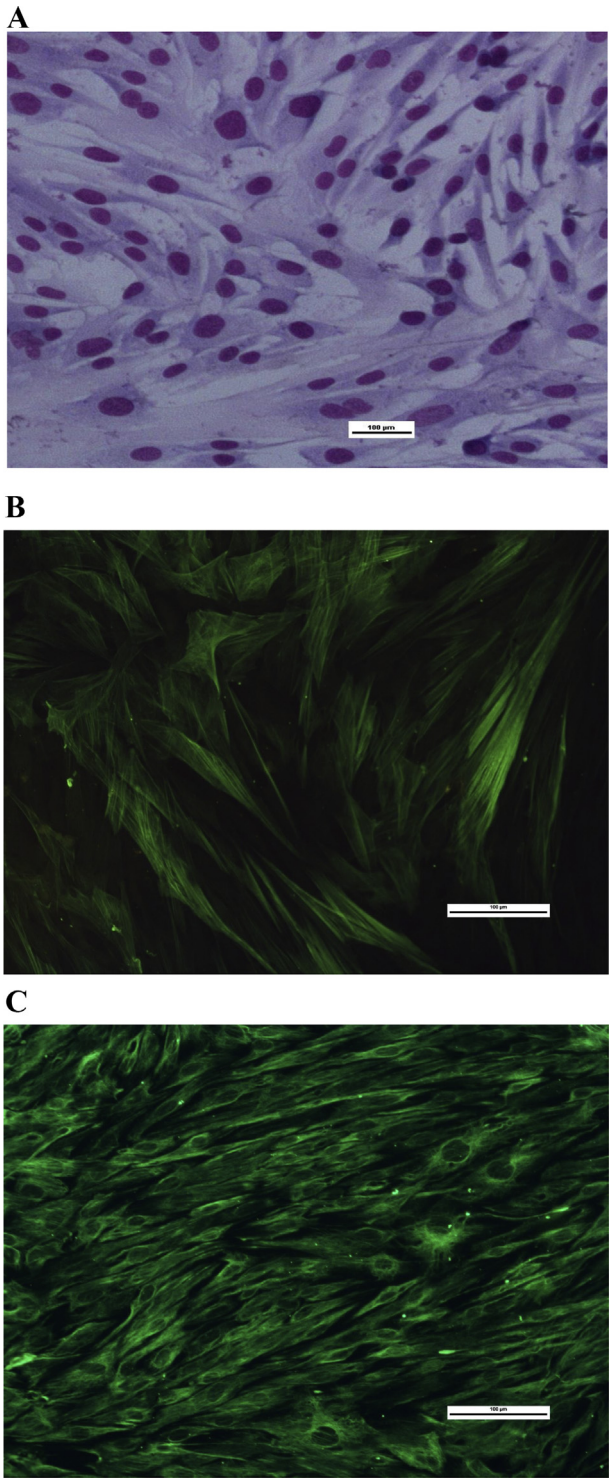

Fig. 4. Identification of dermal papilla cells (DPC) from Rex rabbit. DPC were isolated form 30-d-old Rex rabbit and cultured in vitro. The third generation of DPC were characterized by giemsa staining to observe (A) the cell metamorphosis (100× magnification), and by immunofluorescence technique for (B) the expression of α smooth muscle actin (α-SMA; 200× magnification), by immunofluorescence technique for (C) the expression of versican (200× magnification). Scale bars = 100 μm.

12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels prior to electrophoretic transfer to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA). Standard markers for protein molecular masses were purchased from Thermo (USA). The membranes were blocked with 5% skim milk in PBS (Solarbio, China) at 4 °C overnight and incubated with a 1:1,000 dilution primary antibodies (tubulin AT819, Beyotime, China; phospho-CTNNB1-S552 pAb, Abcam, US; phospho-GSK3B–S9 pAb, Abcam, US; phospho-AKT1-S473 pAb, Abcam, US; or noggin (NOG) polyclonal antibody, Abcam, US). The membranes were then washed with Tris-buffered saline containing Tween (TBST; Solarbio, China) and incubated with a 1:3000 dilution of a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Beyotime, China) at 37 °C for 1 h. The proteins were visualized using Beyo ECL reagents (Beyotime, China) as the reference (Liu et al., 2020b). The intensity of the bands was quantified with a Pro Plus 6.0 Biological Image Analysis System.

2.3. Statistical analysis

All the data were analysed by one-way ANOVA with SAS software (SAS version 8e; SAS Institute, Cary. NC, USA) to determine the significance of differences in the effects of the pyridoxine treatments on the responses among the various groups, Duncan's test was used for multiple comparisons. The data are shown as the means and root mean errors (RMSE), and $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Effects of dietary pyridoxine supplementation on hair follicle development in rex rabbits

Dietary pyridoxine supplementation significantly affected dry matter intakes, total follicle density, secondary follicle density and the secondary-to-primary ratio (S/P, $P < 0.05$). With increasing levels of supplementation, the dry matter intakes and follicle density first increased and then decreased, and maximum values were observed in the 20 mg/kg group (Table 3; Fig. 1). Dietary pyridoxine supplementation increased the gene expression of inositol polyphosphate phosphatase like 1 (*Inppl1*), a component of the PI3K/Akt signalling pathway. The expression *Wnt10b* and *CTNNB1*, components of the Wnt signalling pathway, was increased and the expression of *GSK-3β*, a component of the Wnt signalling pathway, was decreased by dietary pyridoxine supplementation ($P < 0.05$). Pyridoxine promoted the gene expression of *Notch1, Hes1* and *Hes5*, components of the Notch signalling pathway ($P < 0.05$). There was no effect on the gene expression of *BMP2, BMP4* or transforming growth factor-β (*TGF-β1*), which are components of the BMP signalling pathway ($P > 0.05$; Table 4). Pyridoxine increased the levels of phosphorylated CTNNB1 and Akt and decreased the level of phosphorylated GSK-3β ($P < 0.05$; Fig. 2).

3.2. Effects of pyridoxine on the growth of hair follicles from rex rabbits

To investigate the effects of pyridoxine on hair follicles growth, the whisker hair follicles were separated from 30-d-old Rex rabbits. The results showed that the hair follicles of Rex rabbit tentacles cultured in vitro grew at an average rate of 3.89 μm/d for 144 h, and

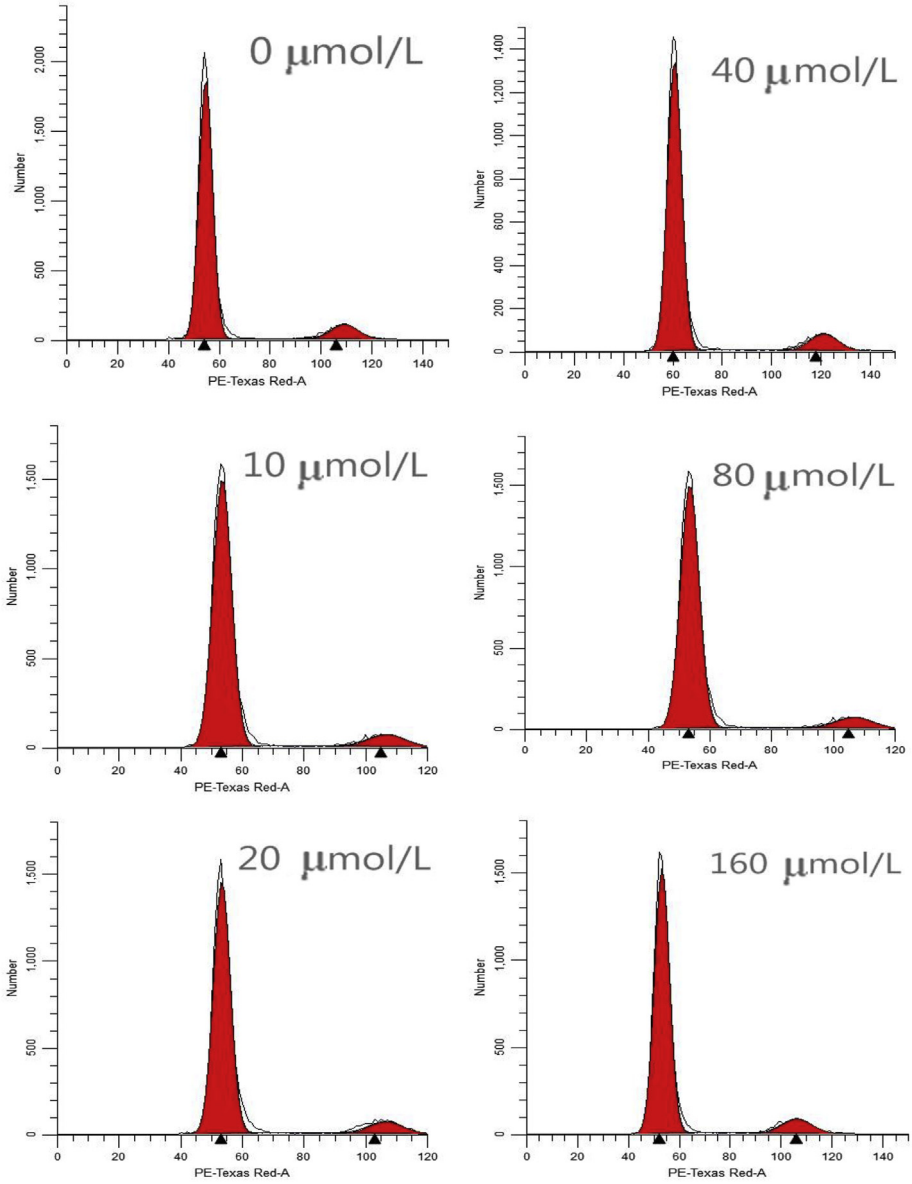

Fig. 5. Effects of pyridoxine on cell cycle of dermal papilla cells (DPC).

the growth rate was faster in the early stage than in the late stage (Table 5). The growth rate of whisker hair stems was promoted by the addition of pyridoxine to the culture medium. The length of Rex rabbit tentacle hair follicles cultured for 144 h reached 36.15 μm in the 80 μmol/L pyridoxine group, with the fastest growth rate of 6.03 μm/d, which was higher than that in the control group ($P < 0.05$; Fig. 3; Table 5). Therefore, pyridoxine could promote hair growth and delay hair follicle degeneration and death in Rex rabbits.

3.3. Effects of pyridoxine on DPC from rex rabbits

To investigate the effects of pyridoxine on hair follicle development, DPC were isolated from 30-d-old Rex rabbits, and these cells were identified by Giemsa staining (Fig. 4A) and by immunofluorescence analysis of the protein expression of α smooth muscle actin (α-SMA; Fig. 4B) and versican (Fig. 4C). The results of the cell proliferation experiment showed that the optical density (OD) values of the 160 μmol/L pyridoxine group were lower than those of the other groups ($P < 0.05$), and the cell cycle progression of the pyridoxine group was different from that of the control group (Fig. 5). The proportion of cells in the resting state/first gap (G0/G1) phase in the pyridoxine group was lower than that in the control group ($P < 0.05$), and the proportion of cells in the second gap/mitosis (G2/M) phase in the pyridoxine group was higher than that in the control group ($P < 0.05$). Pyridoxine at appropriate concentrations (10 and 20 μmol/L) significantly inhibited cell apoptosis ($P < 0.05$), but pyridoxine at high concentrations (80 and 160 μmol/L) significantly promoted cell apoptosis ($P < 0.05$; Fig. 6 and Table 6). Pyridoxine promoted the gene expression of *Inppl1, Inpp4b* and pleckstrin homology like domain family A member 3 (*Phlda3*), which are components of the PI3K/Akt signalling pathway ($P < 0.05$), and *Wnt10b* and *CTNNB1*, which are components of the Wnt signalling pathway, and inhibited the expression of dickkopf-1 (*DKK1*) ($P < 0.05$). The expression of Notch1, Jagged1 *(JAG1), Hes1* and *Hes5*, components of the Notch signalling pathway, was increased by pyridoxine ($P < 0.05$), and the gene expression of *BMP2, BMP4* and *TGF-β1*, components of the BMP signalling

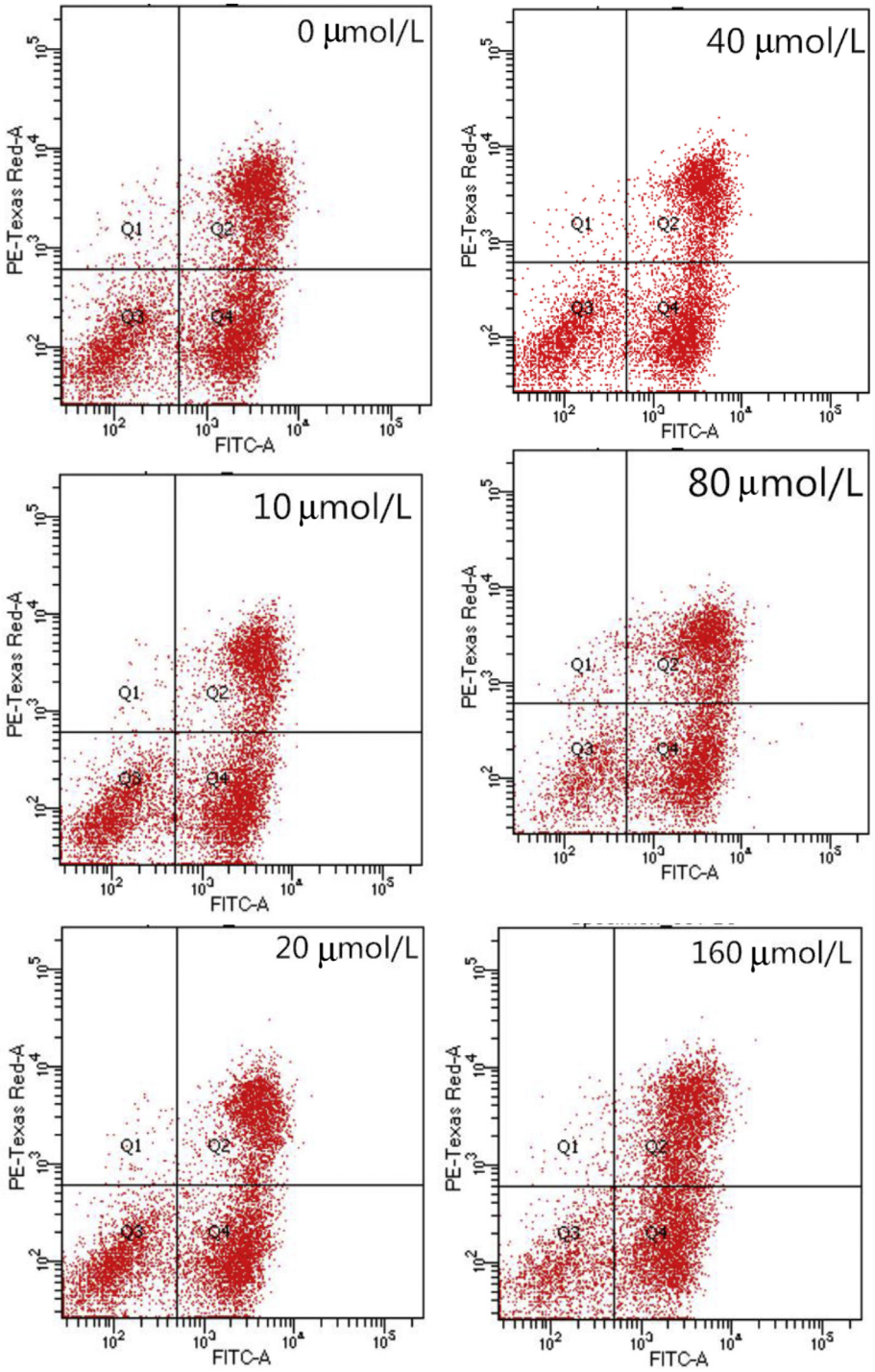

Fig. 6. Effects of pyridoxine on cell apoptosis of dermal papilla cells (DPC).

pathway, was not affected by pyridoxine ($P > 0.05$; Table 7). Pyridoxine promoted the phosphorylation of CTNNB1 and Akt and the protein expression of NOG, inhibiting GSK-3β phosphorylation ($P < 0.05$; Fig. 7).

4. Discussion

Pyridoxine, a form of vitamin B6, serves as a cofactor for enzymes that participate in amino acid metabolism (Jansonius, 1998; Christen and Mehta, 2001; Eliot and Kirsch, 2004; Phillips 2015). Dietary pyridoxine supplementation levels could increase the feed intake of growing Rex rabbits, and the appetite genes of melanocortin 4 receptor and corticotropin-releasing hormone in paraventricular nuclei and peptide YY in duodenum are involved in the pyridoxine-caused hyperphagia (Liu et al., 2015, 2017). Adequate nutrition with higher feed intake could increase the density of hair follicles (Zhu et al., 2020). In this study, dietary pyridoxine supplementation levels increased the dry matter intakes and density of hair follicles of growing Rex rabbits. DPC have potential therapeutic value for the treatment of hair loss and are able to induce the de

Table 6
Effects of pyridoxine on proliferation, cell cycle and apoptosis of dermal papilla cells (DPC)[1].

Item	Pyridoxine concentration, μmol/L						RMSE	*P*-value
	0	10	20	40	80	160		
Proliferation of DPC								
Optical density (OD) value	0.59^{ab}	0.65^{a}	0.52^{b}	0.52^{b}	0.63^{ab}	0.34^{c}	0.111	<0.001
Cell cycle of DPC, %								
Resting state/first gap (G0/G1)	92.09^{a}	91.08^{b}	89.49^{c}	89.92^{c}	89.61^{c}	89.50^{c}	33.641	<0.001
Synthesis (S)	1.99	1.12	1.87	2.02	1.83	0.86	0.729	0.072
Second gap/mitosis (G2/M)	5.91^{c}	7.80^{b}	8.64^{b}	8.06^{b}	8.55^{b}	9.52^{a}	0.609	<0.001
Apoptosis of DPC, %								
Early apoptotic ratio (Q4)	36.34^{a}	31.86^{b}	31.66^{b}	34.95^{ab}	37.88^{a}	38.39^{a}	3.170	0.001
Later apoptotic ratio (Q2)	33.21^{bc}	32.53^{c}	31.40^{c}	36.11^{a}	37.65^{a}	39.13^{a}	3.115	<0.001
Total apoptosis ratio (Q4 + Q2)	69.55^{b}	64.39^{c}	63.06^{c}	71.06^{b}	75.53^{a}	77.51^{a}	4.250	<0.001

$^{a, b, c}$ Within a row, means with different superscript letters are significantly different ($P < 0.05$).
[1] Data represent means and root mean error (RMSE), $n = 8$ for per group.

Table 7
Effects of pyridoxine on gene expression of signal pathway of dermal papilla cells (DPC)[1].

Gene	Pyridoxine concentration, μmol/L						RMSE	*P*-value
	0	10	20	40	80	160		
PI3K/Akt signal pathway								
Inppl1	1.00^{c}	3.46^{a}	3.55^{a}	2.93^{ab}	1.54^{bc}	1.60^{bc}	1.361	0.001
Inpp4b	1.00^{b}	3.91^{a}	2.24^{ab}	1.31^{b}	1.94^{b}	1.20^{b}	1.732	0.018
Frk	1.00	0.80	0.98	0.71	1.14	0.79	0.382	0.231
Phlda3	1.00^{c}	1.68^{b}	1.87^{ab}	1.67^{b}	1.42^{bc}	2.27^{a}	0.503	<0.001
Wnt signal pathway								
Wnt10b	1.00^{b}	1.12^{b}	1.51^{ab}	2.54^{a}	2.22^{a}	2.03^{ab}	0.957	0.012
CTNNB1	1.00^{c}	2.50^{a}	2.20^{ab}	2.60^{a}	1.29^{bc}	1.51^{abc}	1.073	0.017
GSK-3β	1.00	1.67	1.86	1.64	1.83	1.79	0.636	0.094
DKK1	1.00^{a}	0.51^{b}	0.52^{b}	0.16^{c}	0.51^{b}	0.47^{b}	0.453	0.002
Notch signal pathway								
Notch1	1.00^{c}	2.82^{ab}	2.75^{ab}	2.66^{ab}	1.66^{bc}	3.42^{a}	1.278	0.006
JAG1	1.00^{b}	3.17^{a}	3.26^{a}	2.60^{a}	2.66^{a}	3.08^{a}	0.860	<0.001
Hes1	1.00^{c}	1.12^{bc}	0.92^{c}	0.91^{c}	1.89^{a}	1.39^{b}	0.356	<0.001
Hes5	1.00^{c}	1.65^{ab}	1.75^{ab}	1.87^{a}	1.27^{bc}	1.26^{bc}	0.453	0.002
BMP signal pathway								
BMP2	1.00	1.17	0.95	0.75	1.10	0.89	0.403	0.369
BMP4	1.00	0.90	1.15	0.84	1.22	1.06	0.465	0.585
TGF-β1	1.00	1.24	1.09	1.19	0.94	0.88	0.459	0.575

Inppl1 = inositol polyphosphate phosphatase like 1; *Frk* = fyn-related kinase; *Phlda3* = pleckstrin homology like domain family A member 3; *CTNNB1* = catenin beta 1; *GSK-3β* = glycogen synthase kinase 3 beta; *DKK1* = dickkopf-1; *JAG1* = Jagged1; *BMP* = bone morphogenetic protein; *TGF-β1* = transforming growth factor-β.
$^{a, b, c}$ Within a row, means with different superscript letters are significantly different ($P < 0.05$).
[1] Data represent means and root mean error (RMSE), $n = 8$ for per group.

novo formation of hair follicle structures in both the follicular and a follicular epidermis (Jahoda et al., 1984; Kollar, 1970; Oliver, 1970). The PI3K/Akt signalling pathway can activate the Wnt signalling pathway by increasing the phosphorylation of β-catenin and inhibiting the phosphorylation of GSK-3β (Lin et al., 2015). DKK1 can inhibit the phosphorylation of β-catenin, regulate the Wnt signalling pathway and lead to hair follicle regression (Greco et al., 2009). However, when the Notch receptor is bound by its ligands, it can activate hair follicle stem cells and then promote the progression of hair follicles from the resting stage to the growing stage (Demehri and Kopan, 2009). The BMP signalling pathway is involved in embryonic skin appendage organ morphogenesis and postnatal hair follicle growth (Kulessa et al., 2000). The *BMP2* and *BMP4* genes inhibit hair follicle development and are associated with maintaining hair follicle quiescence (Su et al., 2008). Noggin acts as an inhibitor of the BMP signalling pathway, and abnormal Noggin expression leads to follicular enlargement (Zhang et al., 2006). The Notch signalling pathway interacts with the BMP signalling pathway, which can inhibit the Wnt signalling pathway by regulating β-catenin (Demehri and Kopan, 2009). In the present study, pyridoxine significantly affected the gene expression of the PI3K/Akt, Wnt, Notch and BMP signalling pathways in skin tissues and DPC of Rex rabbits, promoted the phosphorylation of CTNNB1 and Akt, and inhibited the phosphorylation of GSK-3β. Therefore, the molecular mechanism by which pyridoxine promotes hair follicle density in Rex rabbits probably occurs through the activation of the PI3K/Akt, Wnt and Notch signalling pathways, prolonging hair follicle growth and delaying the onset of telogen.

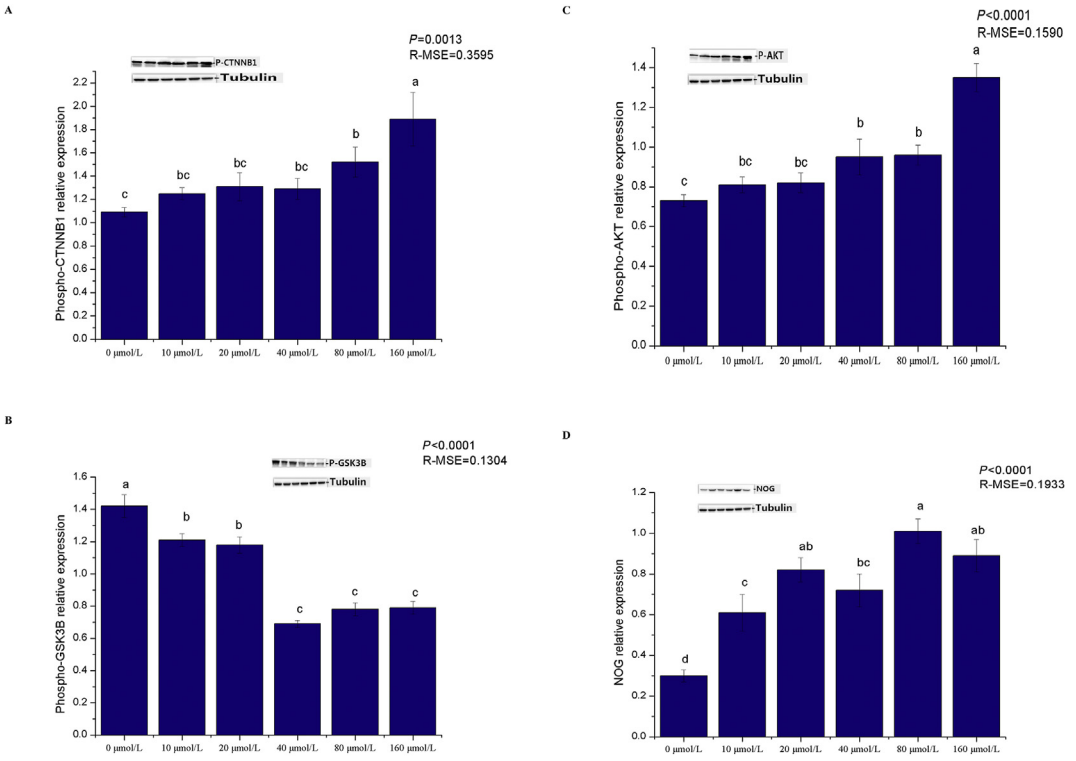

Fig. 7. Effects of pyridoxine on expression of hair follicle-related proteins of dermal papilla cells (DPC). (A) Phospho-catenin beta 1 (p-CTNNB1); (B) phospho-glycogen synthase kinase 3 beta (p-GSK-3β); (C) phospho-Akt (p-Akt); (D) noggin (NOG) protein. Data represent means ± standard error and root mean error (R-MSE), $n = 8$ for per group. [a, b, c] Bars with different letters means significantly different ($P < 0.05$).

5. Conclusion

The molecular mechanism by which pyridoxine promotes hair follicle density in Rex rabbits probably occurs through the activation of the PI3K/Akt, Wnt and Notch signalling pathways, prolonging hair follicle growth and delaying the onset of telogen.

Author contributions

G. Liu and **F. Li** performed the experiments. **G. Cheng**, **S. Gao** and **L. Bai** conceived the project idea, designed the study and supervised the project. **G. Cheng** and **H. Sun** raised the rabbits and conducted the animal experiments. **G. Liu**, **Y. Zhang**, **S. Li**, **Y. Zhu** and **C. Wang** performed the laboratory work. **G. Liu** analyses the data and wrote the manuscript. All the listed authors have read the manuscript and agreed to all of the contents.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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