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Investigation of the Role of Biotin in the Regulation of Wool Growth in Sheep Hair Follicles Cultured *in vitro*

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Abstract: An experiment was conducted to develop an *in vitro* technique to study the role of biotin in controlling growth and viability of sheep wool follicles. Small samples of skin wee taken from mid-rib are of three Suffolk wether lambs and after discarding epidermal layer, anagen secondary wool follicles were isolated from the dermal layer and maintained in William's E Medium supplemented with 0.0,0.25 or 0.5 mg L^{-1} biotin. The results showed significantly increase *in vitro* wool shaft elongation up to 144 h culture in media. There was no significant differences between rate of fibre elongation of viable cultured follicles. Biotin supplementation significantly increased the proportion of follicles remaining viable after 72 h with the order of response of 0.5>0.25>0.0 mg L^{-1} supplement. ATP concentration in follicles maintained in the medium containing 0.50 mg L^{-1} biotin were significantly ($p<0.01$) higher than those maintained in medium containing 0.0 or 0.25 mg L^{-1}. Hair follicles maintained in the presence of 0.5 or 1.0 mg L^{-1} biotin exhibited a significantly ($p<0.01$) higher incorporation of [U-^{14}C] Leucine into protein than in follicles in unsupplemented media. DNA concentration per follicle was not affected by biotin concentration. Visual examination of the bulb of hair follicles confirmed the occurrence of mitotic bodies in the matrix of follicles and indicated the presence of viable cells in growing follicles after 144 h of incubation. An examination of cell proliferation sites, using Brdu, in the hair follicles maintained in the presence of 0.0 or 0.50 mg L^{-1} biotin showed that majority of mitotic activity was concentrated in the bulb adjacent to dermal papilla and outer root sheath in fresh and growing follicles, up to 72 h incubation in different treatments.

Key words: Biotin sheep, follicle, ATP, brdu, DNA, wool growth

INTRODUCTION

The anagen wool follicle is a complex biological system and the production of wool, as in other hair fibre, is a two stage process. Firstly, the proliferative phase involves cell reproduction in follicle bulb. Secondly, the differentiation and keratinisation phase and the production of a keratinized fibre of low moisture content (Schinckel, 1961). However, the mechanisms controlling hair growth are unlikely to be alike in all follicles (Messenger, 1993). Hair growth is cyclical and factors that regulating hair growth cycle are yet poorly understood (Philpott and Kealey, 1994).

The major factors which influence animal fibre growth are genetic potential, nutrition and environment. Results of *in vivo* studies have shown that fibre growth is dependent on the supply of nutrients (including energy and amino acids) to fibre follicles (Galbraith 1998). Nutrient supply to the sheep follicle markedly increased the volume of the germinative region and also the number of bulb cells proliferating per unit time in the wool follicle bulb (Hynd, 2000). The response of fibre growth to energy, protein and sulphur containing amino acid has been investigated *in vivo* and *in vitro* by several authors (Reis *et al*., 1992; Hynd, 2000).

Other nutrients such as fat, vitamins and minerals are also important for normal regulation of hair growth, since it has been shown that low fat diets and fat-free diets can cause alopecia (Bonjour, 1991).

Deficiency of a number of vitamins, especially those of the B-group, may lead to impaired hair growth, probably because these vitamins are co-enzymes in the metabolic sequences and unlikely to have any direct action on the hair follicles (Noppakun and Swasdikul, 1986).

Biotin is a water-soluble B vitamin and functions as an essential cofactor for four carboxylases that catalyse the incorporation of cellular bicarbonate into the carbon

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backbone of certain organic compounds (Bonjour, 1991). Deficiency of this vitamin causes alopecia and hair loss, thinning of hair, loss of hair colour, claw and skin lesion, inflammation of mucous membrane and retardation of growth in human and animals (Mock, 1996; Whitehead, 1988; Robert and Baggott, 1982). Little is known regarding of the importance and exact mechanism of biotin in the processes involved in hair follicular growth and keratinization. However, more detailed studies are required to properly define its roles.

Regulation of the processes involved in fibre production is generally poorly understood (Bathes *et al*., 1999). This is partly due to the lack of reliable *in vitro* models and a situation improved following the development of such a technique by Philpott *et al*. (1989).

The development of *in vitro* culture systems, where confounding systemic influences were eliminated, has proved useful in elucidating aspects of hair regulation and growth for study of human (Philpott *et al*., 1990) rat (Philpott *et al*., 1992) and goat hair follicles (Ibraheem *et al*., 1994).

The objective of the present study was to develop and extend an *in vitro* technique to investigate the importance of biotin for controlling growth and viability of sheep wool follicles by measuring biochemical parameters such as mitotic activity, ATP production, protein and DNA synthesis.

MATERIALS AND METHODS

Culture media and chemicals: Williams' E medium (minus glutamine and biotin), Phosphate Buffered Saline (PBS), Earle's Balanced Salt Solution (EBSS) and all tissue culture glasswares were obtained from Gibco BRL Co (Paisley, UK). Insulin, hydrocortisone, L-glutamine, penicillin, streptomycin, methyl-thymidine, biotin, activated charcoal, EDTA, 3,5-Diaminobenzoic Acid (DABA), 3-aminopropyltrimethoxy-silaine, 3-3 Diaminobenzidine (DAB), 5-Bromo-2-deoxyuridine (BrdU) solution and deoxyribonucleic acid were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Falcon 24-well multiwell plates were purchased from Becton Dickinson Co (Oxford, UK). Anti-mouse rabbit immunogolobulins-biotin labelled (RAM-B), streptavidin and biotin horseradish conjugate peroxidase (biotin-HRP) were obtained from Dako Co (Cambridgeshire, UK). L-[U-^{14}C]-leucine was supplied by Amersham Life Sciences (Amersham, Buckinghamshire, UK). Schiff's reagent was purchased from Raymond Lamb Co (London, UK). Lumac (Sanco Ltd, Bately and West Yorkshire, UK) supplied the ATP monitoring kit.

Animals: Five wether lambs (Suffolk), approximately one year of age (34.5±2.3 kg) were used for this experiment. During the experimental period the animals were housed indoors in wooden slatted pens with free access to feed and water. The lambs were given a diet consisting of chopped hay and concentrate twice a day at 8.30 a.m. and 4.30 p.m. The ration met the requirement of maintenance plus 70 g liveweight gain/day. The animals were weighed weekly before the morning feed.

***In vivo* methods:** The patch sample method was used to measure differences in the rate of fibre growth. A patch (10×10 cm) was tattooed with a permanent marker on the left side midrib area of each lamb. Wool growth on these patches was carefully and quantitatively sheared with animal clippers. The initial patch sample was taken on day 0 and at four week intervals thereafter.

Mean wool fibre growth was measured by randomly selecting around 120 wool fibres from centre of patch samples of each fibre sample. Fibre length (±1 mm) was measured by fixing individual fibres on a clear perspex ruler with the help of fine forceps.

Mean fibre diameter was determined by randomly choosing around 120 fibres from each fibre sample.

***In vitro* methods:** Studies were conducted in the period of September to February. A small skin sample (1 cm^2) was collected by an approved technique from the midrib area on the flank opposite to that used for the collection of patch samples. Samples were collected under local anaesthesia based on the technique described by Ibraheem *et al.* (1994) for goat follicles. In the laboratory the sample was dissected further into strips of 3×10 mm using a scalpel blade. Each section of tissue was then sectioned transversely at a level immediately below the junction between the dermis and the sebaceous gland and most of the connective tissue was removed. The subdermal tissue, containing the intact lower portion of hair follicles, was submerged in Earle's balanced salt solution/phosphate-buffered saline (2:1) in a petridish. The remaining dermal layer was examined under a binocular microscope and intact wool follicles were removed by pulling gently with fine forceps under a dissecting microscope. Intact and morphologically undamaged isolated follicles were then immediately transferred to a suspension culture in individual wells of multiwell plates wells (2 or 3 follicles/well) containing 500 μL Williams' E medium supplemented with 1 mM L-glutamine, insulin (10 mg L^{-1}) hydrocortisone (10 μg L^{-1}) penicillin (64 mg L^{-1}) and streptomycin (100 mg L^{-1}) (designated Williams' E (s) medium) (Philpott *et al*., 1994).

The biotin concentration of the medium was adjusted by supplementation of the medium with 0, 0.25, 0.50 and 1.0 mg L^{-1} biotin. The wool follicles were maintained, free-floating, in individual wells of multiwell plates at 37°C in an unhumidified atmosphere of 5% CO_2/95% air.

Changes in length of follicles due to hair shaft elongation were measured using a Microtec 200 inverted binocular microscope fitted with an eyepiece measuring graticule. An initial measurement of the follicles was taken at 0 h and then every 24 h up to 144 h. Follicle viability was noted and recorded. A follicle was non-viable when their length remained constant over a 24 h period.

The ATP content of wool follicles was measured using a Lumac Biomass Test kit, based on the luciferin/luciferase assay of ATP (Spielmann *et al*., 1981).

Mitotic activity in sheep follicles was analysed using the method of Ferguson *et al.* (1977).

The protein synthesis of cells around the follicles was monitored in culture by measuring the rate of [U-^{14}C]-leucine incorporation into protein. Single follicles or groups of 2-3 follicles (50-70 follicles per treatment) either fresh or maintained in 500 μL Williams' E (s) medium for 48 h in one of the treatments (0.0, 0.25, 0.50 and 1.0 mg L^{-1} biotin) were used. Fresh or cultured follicles were incubated for 4 h at 37°C in 500 μL Williams' E medium supplemented with 1 μCi mL^{-1} [U-^{14}C]-leucine (specific activity 299 μCi m mol^{-1}). After incubation, follicles were washed three times with PBS containing 10 mM unlabelled leucine and then they transferred to Eppendorf tubes containing 1 mL of homogenisation solution containing 0.1 M EDTA, pH 12.4 and homogenised mechanically for 1 min. Following homogenisation, the tubes were centrifuged for 15 min at 12000×g to precipitate cell debris. A quantity (100 μL) of the supernatant was then removed to a separate Eppendorf tube and stored for DNA assay. The remaining supernatant was removed to new Eppendorf tubes for assay of [U-^{14}C]-leucine uptake. The high molecular weight protein in the remaining supernatant was precipitated by addition of 500 μL of 25% (v v^{-1}) PCA and the samples were incubated at 4°C overnight. The precipitated protein was obtained by filtration under vacuum using 21 mm Whatman GF/C filter paper. The filter paper was washed under vacuum by adding 10 mL of trichloroacetic acid (10% w v^{-1}) and then with 5 mL of 5% (w v^{-1}) trichloroacetic acid. After washing was complete, filters were dried at room temperature by using a washing step of 1 mL ethanol/diethyl ether (1:1, v v^{-1}). Dried filter papers were placed directly into scintillation vials and the radioactivity in the precipitated protein was measured by adding 15 mL of ultima gold scintillation solution and using liquid scintillation spectrometry.

The amount of DNA in sheep follicles was measured as described by Fiszer-Szafarz *et al.* (1981) using a fluorimetric method.

BrdU staining was used for the detection of the pattern of cell proliferation on the basis of DNA synthesis in follicles maintained in the presence of 0.0, 0.50 mg biotin L^{-1} for 72 h. Monoclonal mouse anti-BrdU antibody was used in this method to detect the incorporation of thymidine analogue BrdU into DNA of cells as described by Gratzner (1982). BrdU was detected by immunohistochemical staining using the procedure of Gratzner (1982) with some modification.

Statistical analysis: The data from the *in vivo* study were analysed using one way analysis of variance. The effect of time period on the wool characteristics of each animal (e.g. fibre production, fibre growth and diameter) was assessed using the Minitab statistical package (version 13 for Windows). The results of the *in vitro* study were subjected to analysis using the GLM procedure of Minitab. The treatment and animal effects were assessed based on the following model for two way classification (Snedecor and Cochran, 1980):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \epsilon_{ijk},$$

Where Y_{ijk} is the observation on kth follicle of jth animal j (j = 1...n) treated with a ith level of biotin (i = 1...n), μ represents the overall mean, α_i is main effect of treatment (biotin), β_j is the replication (animal) effect and ϵ_{ijk} is the error term.

Comparison of the means were performed using Tukey's pairwise comparisons. Wherever the animal effect was significant, the data were presented in the table as the least square means, otherwise analysis was performed on the observed mean values which were recorded in the table. The data for viability were analysed using Chi-square (χ^2) test using Minitab with comparison between means of the three treatments (biotin concentrations) as follows:

0.0 vs 0.25, 0.0 vs 0.5, 0.25 vs 0.5.

RESULTS

***In vivo* results:** All the animals were in a healthy condition throughout the experiment. The average liveweight gain for all the animals was 75 g per day (± 7.1 S.D).

Fibre yield and characteristic: Mean values for both raw and clean fibre production, fibre growth (mm per day) and mean value for fibre diameter, are shown in Table 1.

Table 1: Wool production and characteristics from Suffolk cross lambs between November and February (mean±S.E.M)[ϕ]

	Growth period[∓]			
Parameter	Nov. 1/11-29/11	Dec. 29/11-27/12	Jan. 27/12-31/1	Feb. 31/1-27/2
Raw fibre production (mg/day/100 cm^2)	61.6 (±6.22)	66.4 (±5.57)	70.1 (±5.19)	69.04 (±3.98)
Clean fibre production (mg/day/100 cm^2)	49.7 (±4.05)	51.7 (±4.14)	52.3 (±3.94)	50.4 (±2.86)
Fibre growth (mm/day)	0.49 (±0.016)	0.50 (±0.017)	0.46 (±0.009)	0.45 (±0.010)
Fibre diameter (μm)	31.6 (±1.66)	32.2 (±2.03)	33.6 (±1.71)	31.3 (±1.71)

[ϕ] Samples of wool were taken from a 10×10 cm patch. Number of lambs sampled = 5, [∓] Difference between sampling times for all parameters were not statistically significant (p>0.05)

Table 2: The effect of different concentrations of biotin on the rate of viable hair follicle elongation (Least Squares Means±S.E.M) maintained for 144 h[ϕ]

	Concentration of biotin (mg L^{-1})[∓]		
Hours of incubation	0.0	0.25	0.50
0-24	0.09 (±0.005)	0.10 (±0.007)	0.11 (±0.006)
24-48	0.11 (±0.007)	0.12 (±0.009)	0.13 (±0.007)
48-72	0.11 (±0.010)	0.13 (±0.011)	0.12 (±0.008)
72-96	0.13 (±0.015)	0.16 (±0.014)	0.16 (±0.010)
96-120	0.13 (±0.019)	0.15 (±0.016)	0.17 (±0.012)
120-144	0.13 (±0.030)	0.14 (±0.019)	0.17 (±0.014)

ϕ Results expressed as least square means±S.E.M. of increase in length of viable follicles. Measurements were made on 90-100 follicles per treatment initially isolated from three sheep, using 30-35 follicles from each biopsy for each concentration of biotin, [∓]The difference between treatments for hair shaft elongation was not statistically significant (p>0.05)

There were no significant differences between periods of clipping (months) in all parameters. The average clean wool production was 76.63%±3.06 of the weight of raw wool fibre. The mean wool fibre elongation in this experiment was 0.48 mm per day. Mean values for diameter were 31.6, 32.2, 33.6 and 31.3 μm for the clips on days 0, 30, 60 and 90, respectively (Table 1).

***In vitro* results**

Follicle elongation and viability: During the course of the experiment it was possible to isolate 50 intact wool follicles in 2 h from small 3×10 mm strips of skin using fine forceps and scalpel blades. Typical sequential growth of a fibre follicle after incubation in culture medium containing 0.5 mg biotin L^{-1} is shown in Fig. 1.

In the present study, marked changes in follicle morphology were observed during culture. The dermal papilla cells of the hair follicles, which showed no more elongation, were considerably altered. These cells became disorganised, as did the matrix cells adjacent to the dermal papilla and dermal papilla become rounded; the matrix cells apparently keratinized with a blackened bulb. These changes in hair follicle morphology are compatible with those reported for human and the wool follicles (Philpott *et al*., 1990; Bathes *et al*., 1997). However, these

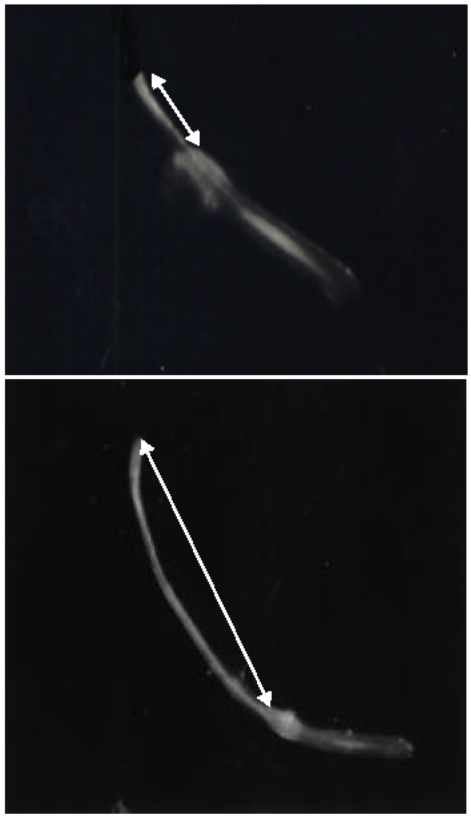

Fig. 1: Light micrograph showing increase in wool follicles length (arrow) with time culture (a) after 24h in culture and (b) after 96 h incubation in Williams' E (s) medium (×25)

morphological changes appeared different to the formation of the club hair-structure in human hair follicles induced by Epidermal Growth Factor (EGF) in culture medium which was reported by Philpott *et al*. (1990) and Harmon and Nevins (1994).

The effect of biotin concentration in the incubation medium on hair shaft elongation and cumulative growth of growing follicles over 144h incubation time periods are presented in Table 2 and Fig. 2, respectively. There was no significant difference in the rate of hair shaft elongation of viable follicles due to biotin concentration, although the animal effect was significant at 48, 72 and 96h incubation.

The effect of biotin concentration in the medium on the percentage of viable follicles over these time periods is shown in Table 3. There was no significant difference between treatments in the percentage of growing follicles up to 48 h incubation time. However, after 48 h, the percentage of viable follicles cultured in media containing 0.0 mg L^{-1} biotin was significantly lower than the percentage of viable follicles cultured in medium containing 0.25 mg L^{-1} or 0.50 mg biotin L^{-1} ($p<0.05$ and

Table 3: The effect of biotin concentrations on percentage of follicles remaining viable during incubation[ϕ]

	Concentration of biotin (mg L^{-1})			$\chi^{2\,\Psi}$		
Hours of incubation	0.0	0.25	0.50	0.0 *vs* 0.25	0.0 *vs* 0.5	0.25 *vs* 0.5
0-24	83	85	90	(0.149) NS	(2.10) NS	(1.14) NS
24-48	77	76	86	(0.028) NS	(2.68) NS	(3.25) NS
48-72	43	59	71	(5.12) *	(15.99) ***	(3.16) NS
72-96	31	49	60	(6.75) **	(16.96) ***	(2.44) NS
96-120	20	40	51	(9.52) ***	(20.99) ***	(2.44) NS
120-144	7	23	32	(10.04) **	(19.91) ***	(2.03) NS

[ϕ] Results expressed as percentage of viable follicles. Measurements were made on the average of viable follicles obtained from three sheep, [Ψ]Comparison of means made by χ^2 test. Figures in the brackets are χ^2 value (d.f = 1); * = p<0.05, ** = p<0.01 *** = p<0.001, NS = Not Significant

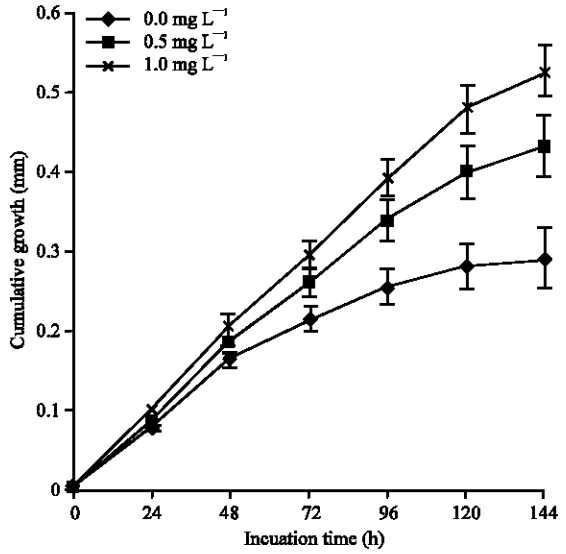

Fig. 2: The effect of different concentrations of biotin on the cumulative growth of isolated secondary wool follicles *in vitro*. Results expressed as least square means±S.E.M. of increase in cumulative growth of total (viable and quiescent) follicles. Measurements were made on 90-100 follicles per treatment initially isolated from three sheep (30-35 follicles from each sheep for each concentration)

p<0.001, respectively). However, the difference between two level of biotin-supplemented (0.25 vs 0.50 mg L^{-1}) group, in all periods, was not significant (p>0.05).

Follicle ATP concentration: The ATP concentration of freshly isolated and growing follicles cultured for 72 h in Williams' E (s) medium containing 1.0 mg L^{-1} biotin are shown in Table 4. ATP concentration in follicles at the time of initial preparation was significantly (p<0.001) lower than those cultured for 72 h. Comparison of the effect of different levels of biotin supplementation after 72 h incubation shows that ATP concentrations in follicles maintained in medium containing 0.50 or 1.0 mg L^{-1} were significantly higher than in those maintained in medium containing 0.0 or 0.25 mg L^{-1} (Table 5). The differences between 0.0 *vs* 0.25 and 0.5 *vs* 1.0 mg L^{-1} biotin were not

Table 4: Mean ATP concentration (mean±S.E.M) in freshly isolated follicles and follicles incubated for 72 h in medium containing biotin at concentrations of 1.0 mg L^{-1}[ϕ] (number of animals = 3)

	Freshly isolated follicles[Ψ]	After 72 h incubation in medium containing 1.0 biotin (mg L^{-1})[φ]
ATP concentration (pg follicle^{-1})[#]	76.8[a] (±34.6)	820[b] (±37.7)

[ϕ] Experiments were carried out as described in the text. Results are given as observed means±S.E.M. [Ψ] For each animal 6-7 samples were assayed (each sample contained 4 follicles, 76 follicles in total). [φ] For each animal 5-6 samples were assayed (each sample contained 4 follicles, 64 follicles in total). [#] Statistical analysis of data was made using one way analysis of variance (unbalanced data). Values with different superscripts are significantly different (p<0.001)

Table 5: ATP concentration (mean±S.E.M) in sheep follicles incubated for 72 h in medium containing biotin at concentrations of 0, 0.25, 0.5 or 1.0 mg L^{-1}[ϕ] (number of animals = 3)

	Biotin (mg L^{-1})[Ψ]			
	0.0	0.25	0.50	1.0
ATP concentration (pg^{-1} follicle)[φ]	385[a] (±46.6)	537[a] (±56.2)	808[b] (±43.9)	820[b] (±46.6)

[ϕ] Experiments were carried out as described in the text. Results are given as observed mean±S.E.M. [Ψ] For each animal 4-6 replicates were assayed (each assay contained 4 follicles), [φ] Statistical analysis of data was made using one way analysis of variance (unbalanced data). Values with different superscripts are significantly different (p<0.01)

significant (p>0.05). There was no significant differences between animals (p>0.05).

Mitotic activity: Photographs of the bulb of whole hair follicles show the presence of viable epidermal cells in the region close to the dermal papilla as indicated by the presence of mitotic bodies associated with cell division (Fig. 3).

Figure 3 shows mitotic activity in a follicle freshly isolated (a) and following incubation for 72 h (b) and 144 h (c) in Williams' E (s) medium containing 0.5 mg L^{-1} biotin (normal medium). In all photographs the presence of mitotic spindles can be observed, indicating replication and separation of the chromosomes. The occurrence of mitotic bodies in Fig. 3-5 (b) clearly indicates the presence of viable cells capable of division after 144 h of incubation. Using this technique it was not possible to quantify differences in mitotic activity in the bulb of whole follicles grown in biotin-deficient (-B) or biotin-supplemented (+B) medium.

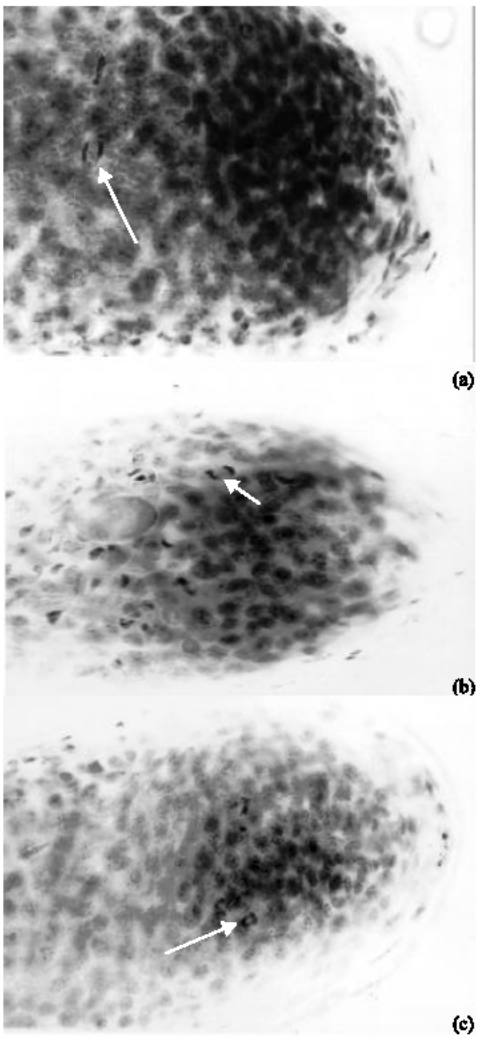

Fig. 3: Matrix region of hair follicle showing mitotic figure (arrow) in a freshly isolated follicle(a) and mitotic activity (arrow) in follicles cultured for 72 h (b) and 144 h(c) in normal Williams' E (s) medium (× 400)

DNA concentration in wool follicles: DNA concentration and [U-^{14}C]-leucine uptake (per follicle and per µg DNA) in freshly isolated follicles and follicles maintained *in vitro* for 48 h are shown in Table 6. As shown, [U-^{14}C]-leucine uptake per follicle in freshly isolated follicles was significantly ($p<0.05$) higher than that in follicles maintained for 48 h in biotin adequate medium. Although DNA concentration and [U-^{14}C]-leucine uptake per µg DNA in fresh follicles were slightly higher than in cultured follicles, the differences were not significant ($p>0.05$).

Table 7 shows DNA concentration and incorporation of [U-^{14}C]-leucine per follicle and per µg DNA in follicles maintained for 48 h in media containing different concentrations of biotin. There were significant differences between levels of biotin supplementation for [U-^{14}C]-leucine uptake per follicle while no significant differences were found for DNA concentration and [U-^{14}C]-leucine uptake per µg DNA. Follicle DNA concentration tended to increase as the concentration of biotin in the incubation medium increased. The uptake of [U-^{14}C]-leucine per follicle increased significantly with increasing biotin concentration. However, the animal effect for these parameters was not significant ($p>0.05$).

Table 6: DNA concentration and [U-^{14}C]-leucine incorporation (mean± S.E.M) into fresh follicles and follicles incubated for 48 h in media containing biotin at concentration of 0.5 mg L^{-1} $^{\Phi}$

	Fresh	48 h $^{\Psi}$
DNA concentration (ng follicle^{-1})	114^{a} (±16.0)	71.9^{a} (±14.8)
[U-^{14}C]-Leucine uptake/follicle (pmol 4 h^{-1})	15.3^{a} (±2.5)	7.2^{b} (±2.2)
[U-^{14}C]-Leucine uptake (pmol µg^{-1} DNA)	136^{a} (±21.9)	116^{a} (±20.3)

$^{\Phi}$ Fresh or cultured follicles for 48h in Williams' E (s) medium, incubated for a further 4 h with 1 µCi mL^{-1} [U-^{14}C]-leucine (specific activity 299 mCi/mmol). Values are the mean of seven assays±S.E.M. (each assay contained 10 follicles; 4 sheep were used, 2 samples for 3 animals and one sample for the 4th animal, 70 follicles in total per treatment), $^{\Psi}$Statistical analysis of data was carried out using one way of analysis variance, using GLM. Values in the same row with different superscripts are significantly different ($p<0.05$)

Table 7: DNA concentration and [U-^{14}C]-leucine uptake into sheep follicles pre-incubated for 48 h in medium containing biotin at concentrations of 0, 0.25 or 0.5 mg L^{-1} and incubated for a further 4 h with 1 µCi mL^{-1} [U-^{14}C]-leucine$^{\Phi}$

	Biotin concentration (mg L^{-1}) $^{\Psi}$			
	0.0	0.25	0.50	1.0
DNA concentration (ng follicle^{-1})	46.6^{a} (±10.4)	52.2^{a} (±9.7)	71.9^{a} (±9.7)	82.5^{a} (±9.7)
[U-^{14}C]-Leucine uptake/follicle (pmol/4h)	4.4^{a} (±1.0)	5.0^{ab} (±0.9)	7.2^{b} (±0.9)	8.9^{c} (±0.9)
[U-^{14}C]-Leucine uptake (pmol µg^{-1} DNA)	93.7^{a} (±11.3)	101^{a} (±10.4)	116.2^{a} (±10.4)	107^{a} (±10.4)

$^{\Phi}$ Experiments were carried as described in the text. Results are given as observed mean±S.E.M of seven assays (each assay contained 10 follicles; 4 sheep were used, 2 sample for 3 animals and one sample for the 4th animal, 70 follicles in total per treatment), $^{\Psi}$Statistical analysis of data was carried out using one way analysis of variance, using GLM. Values in the same row with different superscripts are significantly different ($p<0.05$)

The pattern of cell proliferation: The use of the BrdU probe showed a clearly identifiable population of labelled cells in bulb of follicles which are in mitotic activity. The nuclei in the epidermal cells of the matrix region close to dermal papilla and outer root sheath, reacted with the monoclonal antibody and were stained brown. The pattern of cell division in freshly isolated follicle exposed to BrdU for 24 h with and those maintained for 72 h in either biotin-free or supplemented media, following exposure of the follicles to BrdU for 6 h, is presented in Fig. 3. As it seen in this Fig. 4 (a) in freshly isolated follicles, BrdU is incorporated into the majority of cells with mitotic activity, which is concentrated in follicle bulb adjacent to dermal papilla and also in outer root sheath. The pattern of incorporation of BrdU remained essentially

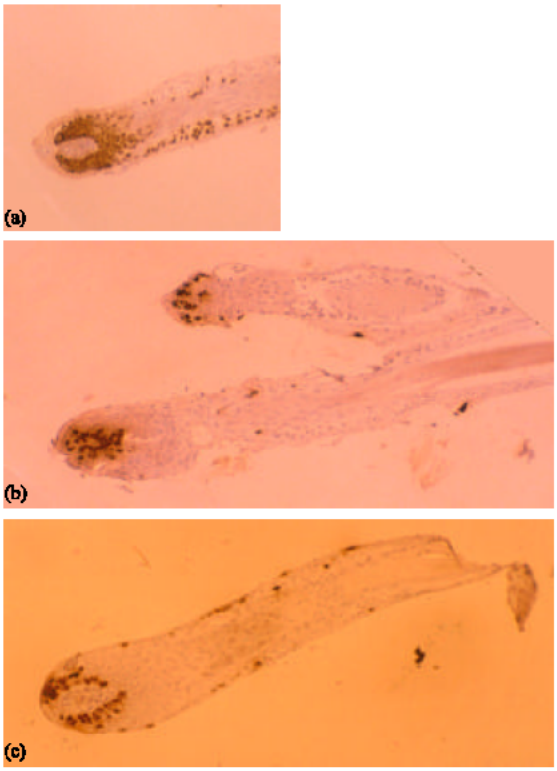

Fig. 4: The pattern of BrdU incorporation following DNA synthesis in wool follicles immunostained by the biotin-streptavidin method; longitudinal section of freshly isolated follicles, showing BrdU uptake by matrix cells adjacent to dermal papilla; (b) hair follicle maintained in biotin-free media for 72 h, with apparently reduced but similar pattern of BrdU incorporation in the matrix as in freshly isolated follicles, (the cells of dermal papilla became disorganised) and (c) hair follicle maintained in biotin-supplemented media for 72 h with BrdU incorporation showing a similar pattern of DNA synthesis (×200)

unchanged after 72 h incubation in biotin-free or biotin-supplemented medium. Marked changes occurred in morphology of follicles cultured for 72 h in biotin-free medium. As it is seen in Fig. 4b the cells of the dermal papilla became disorganised as did the matrix cells adjacent to dermal papilla, compared to those cultured in biotin-supplemented medium Fig. 4c.

DISCUSSION

The regulation of biochemical events in the anagen hair follicle, including fibre production and elongation has been poorly understood. However, it has benefited from the development of *in vitro* tissue culture. Harmon and Nevins (1994) have suggested that requirements of a tissue culture system suitable for the study of hair growth include:

- The maintenance of a high rate of follicular keratinocyte proliferation largely confined to the matrix cells surrounding the dermal papilla.
- The synthesis and post-translational processing of hair fibre proteins, i.e. the elaboration of hair fibre itself.

The isolation of intact hair follicles from human and animal sources by plucking hair from biopsy tissue either using mechanical force or microdissectioning follicles has been conventional methods used for the study of hair tissues (Messenger, 1993).

The *in vitro* study described here is an illustration of successful isolation and growth of hair follicles of sheep, which was initially demonstrated by Ibraheem *et al.* (1994) for Capra hair follicles, using techniques employed by Philpott *et al.* (1990).

In the present in *vitro* study, under sterile conditions, the isolation procedure yields a large number of follicles from the dermis singly or in group of 2 or 3 from the skin sample of Suffolk cross sheep. Under microscopic examination, most of isolated follicles were morphologically intact and undamaged.

In the biopsy of skin it was found that wool follicles in some animals had a curved shape and lie at an angle to the skin surface which results in marked variation in follicle depth as described by Orwin (1989). However, most of follicles lie parallel and straight in the dermal tissue and so isolation of follicles was relatively easier than in other animals.

The mean daily rate in wool shaft elongation in active follicles was observed up to 144 h with a mean value of 0.13 mm day^{-1} and range of 0.05 to 0.475 mm day^{-1} and was significantly less than that for grown *in vivo* (0.48 mm day^{-1}). The lack of certain factors in the media, such as trace elements, co-factors, or hormones and their interaction, may contribute to the explanation for higher fibre elongation *in vivo* than *in vitro*.

However, the values presented in this study are similar to those reported for *in vitro* culture of cashmere hair follicles (0.12-0.14 mm day^{-1}, Souri 1998; Ibraheem *et al.*, 1994) and mohair (0.17-0.20 mm day^{-1}; Souri 1998) but less than the data published by Philpott *et al.* (1990) for human hair follicles (0.3 mm day^{-1}).

Incubation of sheep follicles with different concentrations of biotin showed that the percentage of growing follicles in all treatments declined in each 24 h

measuring period. Significantly greater reduction was found in the percentage of viable follicles following 72 h culture in biotin-free medium in comparison with supplemented media. In an attempt to explain the observed decrease in viability of follicles, it might be possible that some locally produced stimulatory factor becoming limiting or a locally produced inhibitor reaching a threshold (Hynd, 2000). The trauma of isolation of hair follicles and lack of an essential nutrient may also have negative effects on viability of follicles (Ibraheem *et al*., 1994).

It is well established in animals, that deprivation of biotin results in dermatological lesions of hoof and skin and alopecia. However, the mechanisms remain to be elucidated (Mock, 1996). One possibility for the observed reduction in the viability of follicles cultured in biotin-free medium, in the present study, is an increase in concentration of abnormal metabolites in these follicles due to biotin deficiency. It seems that the bulb cells of the follicle may be much more sensitive to toxicity than other types of cells in the skin such as fibroblasts (Frater, 1983). Beemer suggested that alopecia may be due to the accumulation of the abnormal metabolites such as organic acids, for example, 3-methylcrotonylglycine and 3-hydroxyisovaleric acid. These disorders are associated with deficiency of the biotin dependent enzyme, *3-methylcrotonyl-CoA carboxylase*, which is essential for metabolism of leucine. This deficiency results in the shunting of the leucine degradation pathway into abnormal organic acid production (Liu *et al*., 1993). These organic acids may induce toxicity in follicles and so reduce viability of follicles.

Since the growth of some follicles in biotin-free medium was particularly evident after 72 h incubation, it is possible that adequate residual biotin may be present in intracellular stores of some follicles to maintain cell growth and differentiation during the short period of the study. However, in this study concentrations of biotin in follicles were not measured.

In order to observe the metabolic activity of isolated follicles, ATP concentration was assessed in fresh and growing follicles maintained for 72 h in different culture media. ATP concentration was significantly lower in freshly isolated follicles than in cultured follicles (77 vs 820 pg follicle^{-1}). The above result is in accordance with those obtained in mohair (140 vs 917 pg follicle^{-1}, Ibraheem *et al*., 1993). An alternative explanation for the lower ATP concentration in freshly isolated hair follicles than in cultured follicles is that the ATP was used by cells within the 2 h time period prior to analysing ATP concentration (biopsy, isolation, etc.) prior to the production of energy substrate in culture media.

The concentration of ATP in cultured follicles after 72 h was determined in different medium containing different concentrations of biotin. A decrease in the concentration of ATP in follicles cultured in biotin-free medium was observed which closely followed the decline in follicular viability at 72 h incubation. This suggests that ATP concentration may be associated with the maintenance of proliferation and viability of follicles. It is also possible that the observed decrease in the viability of follicles cultured in biotin-free medium may cause the reduction of ATP generation. It has been previously observed that ATP production is associated with glucose utilisation in hair follicles which are actively growing (Johnson, 1977) although only 10% of glucose is oxidised to CO_2 and the remaining 90% is attributed to lactate production in rat hair follicles (Philpott et *al.* 1992).

Furthermore, biotin may be involved as an essential contributor to fatty acid metabolism. Lipids have a functional role in the electron-transfer process and energy generation via structural roles in the mitochondria. Mitochondria also contain varying amount of citric acid cycle enzymes and other enzymes which are involved in electron transport, ATP formation and other metabolic processes in tissues. Approximately one third of the dry weight of mitochondria is lipid (Dakshinamurti and Litvak, 1970) and biotin is an essential co-enzyme for acetyl CoA and hence lipid metabolism. Deficiency of biotin may thus impair lipid synthesis in tissues and mitocondrial membranes. This hypothesis is in agreement with the previously published information (Dakshinamurti and Litvak, 1970). These workers showed that the Respiratory Control Ratio (RCR) rate of respiration and ATP synthesis were significantly reduced in biotin-deficient rat liver cells mitochondria as compared with mitochondria cultured in normal medium. They showed that biotin-deficient mitochondria had a decreased respiratory control ratio in the oxidation of all NAD^+ linked substrates such as β-(OH)-butyrate and pyruvate plus malate. These authors concluded that mitochondrial membranes in biotin-deficient cells might be responsible for these defects and this change might cause structural damage resulting in fragility of the "deficient" mitochondria. An addition, Nakano *et al*. (1982) have suggested that biotin is critical for maintaining the Kreb's cycle as well as generation of ATP, through its role in oxaloacetate formation.

Evidence to support the successful maintenance of wool follicles in the *in vitro* system in the present study was demonstrated by the detection of mitotic activity in cultured follicles. It is well established that only cells surrounding the dermal papilla in the follicle bulb and those of the outer root sheath undergo mitosis

(Louie *et al.*, 1998). The continued occurrence of mitosis in the bulb of freshly isolated sheep wool follicles and those maintained for 72 h in culture indicated the presence of viable follicles throughout the culture. However in histological examination visually, no apparent difference in mitotic activity was found between follicles growing in biotin-free or supplemented medium, because it was not possible to measure mitotic activity by vision.

The histological sections of wool follicles presented in this study, clearly showed that most of the mitotic activity, as expected, occurred around the dermal papilla and outer root sheath. The localisation of BrdU staining and pattern of DNA synthesis was similar in growing follicles maintained for 72 h both in biotin-free or supplemented medium.

The DNA concentration established here for sheep follicles of an average 65 ng per follicles (Table 7) is similar to that reported in mohair (65-76 ng per follicles, Souri, 1998) and rat (75-100 ng per follicles, Philpott *et al.*, 1992).

In the present study, trends were evident which suggested a dose-related response in DNA concentration with increasing biotin concentration in the medium. The concentration of DNA in follicles cultured in biotin-free medium after 48 h (47 ng per follicle) was 35% lower than those cultured in biotin-supplemented (0.5 mg L^{-1}) medium (72 ng per follicle) (Table 7), although differences between treatments were not significant ($p>0.05$). This result may be considered in the context of previous observation of other authors e.g. Sarasin (1994) and Moskowitz and Cheng (1979). Sarasin (1994) reported that, biotin at the pharmacological concentration of 0.244 mg L^{-1}, significantly stimulated cell proliferation, DNA and protein (keratin) synthesis in Outer Root Sheet (ORS) of bovine and swine hair follicles. Similarly Moskowitz and Cheng (1979) showed that biotin deficiency significantly decreased the rate of DNA synthesis in Sarcoma virus-transformed cells cultured in serum-free media. Bhullar and Dakshinamurti (1985) have also provided evidence that the cell cycle in the G1 stage of mitosis (measured by thymidine uptake) was blocked in Hela cells cultured in biotin-deficient medium and the block was removed after addition of biotin into cells in deficient media.

Protein synthesis requires the co-ordinated interplay of more than a hundred macromolecules e.g., RNA, enzymes, ATP, etc. Protein synthesis occurs mainly in the keratogenous zone of the follicles. Further up the follicle, cells reach the zone of hardening where the final processes occur that result in the final formation of over 100 distinct proteins which makes up the wool fibre (Hardy, 1992). Several intermediate filament-associated proteins are synthesised in the hair follicles. These are related but chemically different from those formed in the soft keratin of the epidermis. According to particular features of fibre amino acid composition, hair and wool proteins of growing follicles are categorised into 3 classes: High-glycine/tyrosine protein; high-sulphur or ultra high-sulphur proteins and low-sulphur α-helical fibrillar proteins (Rogers *et al.*, 1989).

Measurement of the effect of biotin in culture media on protein synthesis in the wool follicles, were also made in the present study. The follicles cultured in biotin-supplemented medium exhibited a significantly greater rate of [U-^{14}C]-leucine uptake (pmol) per follicle, than those cultured in biotin unsupplemented medium and the differences were statistically significant ($p<0.05$). However, despite improvements in the incorporation of [U-^{14}C]-leucine (pmol) into protein (per μg DNA) of hair follicles, produced by increasing biotin concentration in the media, differences were not statistically significant.

The observed increase in incorporation of [U-^{14}C]-leucine per follicle due to biotin supplementation, could be attributed to a greater percentage of viable dividing bulb cells in biotin-supplemented follicles, which contribute to fibre viability.

There is some evidence in previous studies that shows the importance of biotin in protein synthesis and in maintaining viability of cells. For example the expression of specific keratins (48 KDa, 56 KDa and 56.5 KDa) of outer root sheet cells of bovine and swine hair follicles was significantly increased when cells were cultured in the presence of biotin (Sarasin, 1994). Pienkowska and Koziorowska (1975) have shown that, compared to biotin-supplemented media, the concentration of aspartic acid in the mouse leukaemic cells grown in biotin-free media was decreased. Furthermore, biotin depletion from culture medium markedly reduced the rates of protein and DNA synthesis of human fibroblasts (Ghneim and Al-Shammary, 1993). Thus, it is suggested that biotin may have been involved in nuclear metabolism. Bhullar and Dakshinmurti (1985) have shown that the number of functional ribosomes of Hela cells grown in biotin-deficient media was decreased. They also suggested that the role of biotin in protein synthesis can be due to either increased rate of translation of performed mRNA or synthesis of new mRNA. This conclusion is supported by the study of other researchers such as Dakshinmurti and Litvak (1970). These researcher observed a 250% increase in the incorporation of orotanic acid 6-^{14}C (intermediate metabolite for synthesis of purines such as uracil) into nuclear RNA when biotin-deficient rats were injected with 200 μg of biotin 20 h before sacrifice. The effect of biotin on mRNA synthesis could also be related to its role in ATP formation via providing energy for this process as described earlier.

On the basis of these results, two conclusions can be drawn. First, the successful culture *in vitro* of sheep follicles provides a model with the potential to study the direct influence of endocrine, nutritional and local factors on wool protein synthesis independently of systemic shifts in the animal's metabolism. The method for the isolation of intact and viable follicles from skin is rapid it is feasible to maintain sheep hair follicles successfully up to 7 days, although during this time there was some loss in viability (68% up to 144h). These data confirm the viability of follicles in the *in vitro* culture system by means of biochemical methods such as DNA synthesis by incorporation of BrdU, [U-^{14}C]-leucine uptake, mitotic activity and ATP synthesis.

Secondly, findings presented in this study provide evidence that biotin plays an important role in the viability and regulation of hair follicles metabolism as it provided the maintenance of viability, increased ATP concentration and the rate of protein synthesis in the cultured hair follicles. However, the mechanism of this effect is not clearly understood.

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