Minoxidil stimulates mouse vibrissae follicles in organ culture

Hair Growth Research (AEB, DJW, TTK), Pathology and Toxicology (JMH), The Upjohn Company, Kalamazoo, Michigan, U.S.A.

Minoxidil, a potent vasodilator, stimulates the growth of terminal hair from vellus or miniaturized follicles in balding scalp. To study minoxidil's action on isolated follicles we developed and validated an organ culture system using mouse whisker follicles. Control follicles cultured without minoxidil showed macroscopic changes including kinking of the hair shafts and bending of the follicles. Necrosis was evident in the differentiating epithelial elements forming the cuticle, cortex, and inner root sheath. These abnormalities were eliminated or greatly reduced in minoxidil-treated follicles. The morphology of these follicles was consistent with the production of new hair during culture. Direct measurement demonstrated that minoxidil-treated follicles grew significantly longer than control follicles during the 3rd culture. Minoxidil increased the incorporation of radiolabeled cysteine and glycine in follicles compared with control treatment. Doses of minoxidil up to 1 mM caused increased cysteine incorporation, while higher doses were inhibitory. Experiments with labeled thymidine indicated that minoxidil induced proliferation of hair epithelial cells near the base of the follicle. Autoradiography also showed that cysteine accumulated in the keratogenous zone above the dermal papilla. These studies demonstrate that organ cultured follicles are suitable for determining minoxidil's mechanism of action and may be useful for studying other aspects of hair biology. The results also show that minoxidil's effect on hair follicles is direct. This suggests that minoxidil's action in vivo includes more than just increasing blood flow to hair follicles. J Invest Dermatol 92:315–320, 1989

Minoxidil is a potent antihypertensive that induces hypertrichosis as a commonly occurring side effect [1–3]. Clinical trials indicate that topical application of the drug causes hair growth in a significant proportion of treated patients suffering from androgenetic alopecia [4]. Studies in both men and transsexualized macaques show that topical application of minoxidil stimulates miniaturized follicles to enter anagen and increases the hair shaft diameter in treated areas [4–6]. The mechanism of minoxidil's action is not known. The drug relaxes vascular smooth muscle and increases cutaneous blood flow. This effect is hypothesized to be responsible for the drug's action in causing increased hair growth [7].

The biology of hair growth is an area in which many basic questions remain unanswered. The question of minoxidil's mechanism of action is but one of these. Others stem from observations that the dermal papilla is a critical component of the hair follicle [8–9]. The nature of the regulatory mechanisms between the dermal and epithelial components of the hair follicle is unknown. An additional area of interest includes the mechanisms that control the hair follicle regression through telogen and reentry into anagen. Critical studies designed to answer these questions require development of an organ culture system in which isolated hair follicles can be studied free of somatic influences. Some reports on development of hair within cultured embryonic or newborn rodent skin are published [10–13]. Only a small number of follicles continue hair production in culture when enzyme isolated coat follicles are cultured [14].

The purpose of the present series of experiments was to develop and validate an organ culture model for studying the biology of hair growth and the action of minoxidil on hair follicles. In this paper we report a method for culturing mouse vibrissae follicles. Minoxidil is active in this system and stimulates both proliferation and differentiation of the matrix cells in cultured follicles. These results suggest that minoxidil has direct effects on anagen follicles which support hair growth.

MATERIALS AND METHODS

Vibrissae were harvested from F1 hybrid mice (C57BL6 × C3H) at 3–4 d of age. These mice were offspring of pregnant mice reared at Upjohn. The neonates were killed by cervical dislocation; then the whisker pads were removed and placed in Hank's balanced salt solution. Follicles were dissected from the whisker pads using jeweler's forceps under a stereo microscope. A total of seven to nine intact follicles from the two ventral-dorsal rows nearest the eye were harvested from each pad. In most experiments, follicles from a single litter of pups were pooled, then assigned to individual treatment groups. Each treatment group consisted of a group of 4–11 follicles cultured together in an individual well of a 24-well tissue culture plate (Corning, Corning, NY). Culture was done in 10% CO2 at 37°C. Dulbecco's Modified Eagles medium (catalog number 320-1965, Gibco, Grand Island, NY) containing 20% fetal bovine serum (Gibco) and 12.5 μg/ml of Gentamicin (Gibco) either with or without minoxidil was used in the experiments. Minoxidil was dissolved in the culture medium at concentrations between 0.05 and 10.0 mM. At the start of each experiment follicles were placed in the minoxidil-containing or control medium and left in that medium through the end of the 48 or 72 h culture period. The minoxidil used for these studies was purified material manufactured by Upjohn.

Growth in culture was assessed by measuring increases in follicle length over a 72 h culture period. The distal end of each hair shaft

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Reprint requests to: Allen E. Buhl, Hair Growth Research, The Upjohn Company, Kalamazoo, MI 49001.
was anchored to 60 mm culture dishes (Corning, Corning, NY) with melted dental wax (Polysciences, Warrington, PA), and photographs of individual follicles were taken both at 0 and 72 h. The distance between the base of the follicle and the wax attachment site on the hair shaft was measured in each photograph, and from these measurements the increase in follicle length was calculated.

Minoxidil stimulation of the follicles was also determined by measuring the incorporation of radiolabeled thymidine and amino acids in the hair matrix during culture. Five μCi/ml of 3H-thymidine, 0.5 μCi/ml of 3H-glycine, or 0.5–5 μCi/ml of 35S cysteine (Amersham, Arlington Heights, IL) was added to the media 24 h after the follicles were placed in culture. After a 4-h rinse in normal saline, the amount of radiolabel uptake was measured by scintillation counting of individual follicles. 3H and 3H/35S labeled follicles were solubilized in 1 ml of Soluene (Packard Instrument Company, Downers Grove, IL), neutralized with 0.1 ml of 1N HCL, and counted in 10 ml of ACS scintillation fluid (Amersham Corporation). Follicles labeled with 35S alone were counted in ACS without solubilization.

Localization of radiolabel was determined by autoradiography. Control and treated follicles were cultured for 24 h, then the follicles were labeled for 8 h by adding 2.5 μCi/ml of radiolabel to the media. The follicles were then fixed overnight in 10% buffered formalin and processed for paraffin embedding. Sections were cut at 4–5 μm thickness, dipped in NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY), and stored in a dark container containing desiccant at 10–14°C at 20°C. The emulsion was developed at room temperature for 2 min in D-19 (Eastman Kodak), rinsed twice in water, then fixed in Kodak fixer (Eastman Kodak) for 2 min. Slides were counterstained with hematoxylin and examined using light microscopy.

To determine minoxidil effects on morphology both macroscopic and microscopic examinations of cultured follicles were done. Minoxidil effects on macroscopic morphology were analyzed in 10 experiments in which radiolabel uptake was also measured. For one of the microscopic studies, pairs of matched minoxidiltr and control follicles were embedded in methacrylate, sectioned at 1.5 μm, then stained with periodic acid Schiff's hematoxylin and eosin stains. Slides produced for the autoradiography experiments were also examined for minoxidil effects on follicle histology.

In both the autoradiography and the histology experiments, independent control groups of follicles were also cultured with and without minoxidil. Radiolabeled cysteine was added to these follicles and the incorporation of label was measured by scintillation counting at the end of the culture period. The minoxidil-induced uptake of 35S-cysteine was used as a positive control to show that the culture system worked.

All experiments were analyzed using analysis of variance. This was followed by least-squares means test or t-tests comparing the control group, without minoxidil, to the minoxidil-treated groups to document minoxidil stimulation. Chi2 analysis of contingency tables was used to determine the effect of minoxidil on follicle morphology. A significance level of p < 0.05 was used throughout these experiments.

RESULTS

Dissection of the follicles yielded follicles relatively free of contaminating connective tissue. Use of neutral red dye showed that the follicles were viable in culture for 10 d, the longest period tested. Follicles cultured for longer than 3 d began to lose the morphologic distinctions between the various tissue layers and showed decreasing outgrowth of cells on the bottom of the culture wells.

Culture of follicles for 48 h without minoxidil altered the gross morphology of the follicles, while culture with 1 mM minoxidil maintained normal morphology (Fig 1). Control follicles were bent and the hair shafts within the follicles were kinked. Also, the area of pigmented matrix cells near the dermal papilla was thinner and more poorly defined than in freshly dissected or minoxidil-treated follicles. Follicles cultured with minoxidil appeared similar to the fresh follicles but had an obvious outgrowth of root sheath along the hair shaft. The frequency of morphologic changes was significantly affected by minoxidil (Table I).

Histologic sections of representative control and treated follicles cultured for 72 h clearly show the effects of minoxidil (Fig 2). Seventy percent of control follicles showed evidence of necrosis in the differentiating epithelial elements of the hair matrix. Cells with pyknotic nuclei, large vacuoles, and other indicators of degenerative processes were found in matrix elements that form the cuticle, cortex, and inner root sheath. The frequency of these abnormalities was significantly reduced in minoxidil-treated follicles (Table II). The morphology of 86% of the minoxidil-treated follicles was consistent with the production of new hair during culture.

Minoxidil stimulated both 35S-cysteine uptake and an increase in follicle length (Fig 3). Over the 72-h culture period, follicles incubated with 0.5 mM minoxidil increased in length as compared with follicles cultured without minoxidil. Measurement of these same follicles also demonstrated that minoxidil increased uptake of radiolabeled cysteine.

The minoxidil stimulation of cysteine uptake in cultured follicles showed a dose response relationship (Fig 4). Minoxidil at 0.05 mM caused no increase in 35S-cysteine, and both 0.5 and 5.0 mM in-
Table I: Changes in the Gross Morphology of Vibrissae Follicles Following 3 d in Culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>Altered Morphology</th>
<th>Normal Morphology</th>
<th>Altered Morphology</th>
<th>Normal Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>164</td>
<td>146</td>
<td>18</td>
<td>89</td>
<td>11</td>
</tr>
<tr>
<td>Minoxidil</td>
<td>157</td>
<td>7</td>
<td>150</td>
<td>4</td>
<td>96</td>
</tr>
</tbody>
</table>

* Follicles in a series of 10 consecutive experiments were rated as having either normal or altered morphology. Normal follicles were similar to freshly dissected follicles with no kinks or other abnormalities in the pigmented hair matrix. Altered follicles were bent with kinks in the differentiating hair matrix. Minoxidil treatment significantly altered the morphology of these cultured follicles, p < 0.05.

Increased uptake, while 10.0 mM had no effect. Minoxidil at 0.5 mM increased the incorporation of $^3$H-glycine as well as $^{35}$S-cysteine (Fig 5).

Minoxidil stimulated follicles even when addition of the drug was delayed until 24 h after the start of the culture (Fig 6). Minoxidil treatment from the start of culture (0 h) through 24 h caused no stimulation of cysteine uptake in the minoxidil follicles compared with controls. As expected, follicles incubated with minoxidil from 0–48 h showed a clear minoxidil effect on cysteine incorporation. Following a 24-h period without minoxidil, a subsequent 48-h minoxidil treatment (24–72 h) also resulted in a significant stimulation of cysteine uptake. The magnitude of cysteine uptake in this group was similar to that of the minoxidil group without the 24-h delay in minoxidil treatment.

Increasing concentrations of minoxidil also increased thymidine uptake (Fig 7). Although 0.25 mM was ineffective, both 0.5 and 1.0 mM minoxidil stimulated thymidine uptake during a 48-h incubation.

 Autoradiographs of $^{35}$S-cysteine labeled follicles showed that the localization of the radiolabel was primarily in the keratogenous zone distal to the dermal papilla (Fig 8). In the presence of minoxidil, grains were present in the germinal epithelium and more grains were found in the keratogenous zone. Preliminary results with computer image analysis indicated that grain density of the differentiating epithelial cells (inner root sheath through medulla) increased 2–3 times in minoxidil-treated compared with control follicles. Control follicles cultured either with minoxidil-containing or control media were run in these experiments and showed the expected minoxidil-induced increase in cysteine.

Studies with $^3$H-thymidine showed that it was localized primarily in the matrix cells below Auber's critical line (Fig 9). Follicles grown in the presence of minoxidil showed increased numbers of labeled nuclei. Labeling of nuclei in a few cells of the outer root sheath was observed regardless of the presence or absence of minoxidil. Nuclei of the inner root sheath cells were not labeled except for

Figure 2. Micrographs of follicles cultured for 72 h in control media (a) or media containing 0.5 mM minoxidil (b). The arrow indicates the area of necrotic hair matrix in the control follicles. Bars: 0.01 mm.

Figure 3. Minoxidil effects on change in follicle length and cysteine uptake. follicles were photographed at the beginning and end of the 72 h culture period and the change in length was calculated from measurement of the photographs. The follicles were cultured in $^{35}$S-cysteine (0.5 μCi/ml), and uptake of this label was measured at the end of the culture period. Counts are per follicle; bars represent means ± SEM; and asterisks indicate a significant difference from control group, p < 0.05.
Table II. Histology of Vibrissae Follicles Cultured for 3 d

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Follicles</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Altered Morphology</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Minoxidil</td>
<td>28</td>
<td>4</td>
</tr>
</tbody>
</table>

*Follicles in five experiments were classified on the basis of their microscopic morphology. Follicles classified as normal had no defects, while those classified as altered showed degenerative changes in the elements of the hair matrix. Only follicles in which a good sagittal section was obtained were included in this analysis. Minoxidil treatment significantly altered the degenerative changes in these cultured follicles, p < 0.05.

a few nuclei in the distal end near the skin-follicle interface. No labeling was seen in the dermal papillae under any conditions. Uptake of 51H-thymidine was measured by scintillation counting in both control and minoxidil-treated follicles in these studies and showed the expected minoxidil-stimulated increase in radiolabel uptake.

DISCUSSION

Both our macroscopic and microscopic analysis show that minoxidil treatment of follicles in vitro tends to maintain normal follicle morphology. Culture conditions usually do not accurately mimic the complex in vivo environment and thus alter the normal complex coordination of growth and differentiation. This is shown by the necrosis of the matrix cells and the kinking of the follicles cultured without minoxidil. Minoxidil treatment normalizes follicle morphology, reduces necrosis, and stimulates proliferation of both matrix and inner root sheath cells. Minoxidil clearly stimulates hair follicles cells in culture.

Several other groups of investigators have used organ culture techniques to culture hair follicles from rodents with mixed success. Histologic analysis of embryonic vibrissae in vitro shows a similar developmental profile as follicles in vivo [10]. Recombinant studies with fetal dermis and epidermis indicate that the development of coat follicles can be maintained up to the stage that hairs emerge from the follicles [11]. Continued growth of postembryonic coat follicles is reported in explants embedded in a collagen matrix and incubated with an extract of embryonic mice [12]. Culture of isolated coat follicles in collagen supports the cells in the outer layers of the follicles, but there is little evidence of continued growth and differentiation of matrix cells [14].

The success of our studies in demonstrating hair growth in vitro depends upon careful dissection of intact anagen follicles as complete follicles containing all the cell layers necessary for the differentiated process of hair growth. Untreated follicles show little, if any, hair growth or incorporation of metabolic labels in culture. Apparently, additional factors and/or special conditions are present in vivo that maintain follicle growth. Under our culture conditions, minoxidil supplementation is essential to continued performance and reinitiation of follicular function under these adverse conditions.

We base our use of radiolabeled cysteine as a measurement for hair growth on reports that cysteine is a major component of hair proteins in mice [15]. Radiolabeled cysteine is used to successfully quantify hair growth in vivo [16]. Our data showing that minoxidil also stimulate the incorporation of glycine indicate that the increased uptake of amino acids is not unique to cysteine. We have experiments in progress to determine which specific proteins incorporate the radiolabeled amino acids. In the vibrissae cultures, radiolabeled cysteine is located in the keratinogenic area of the follicle, as expected. Thus we conclude that minoxidil stimulates, or at least supports, differentiation of hair keratinocytes.

The thymidine uptake data support the conclusion that minoxidil has a mitogenic effect on the matrix cells of hair epithelium. This proliferative effect is in the area of the follicle below the widest part

![Figure 4](image1.png)

**Figure 4.** Dose response of minoxidil on cysteine uptake in follicles cultured for 72 h. Follicles were labeled with 35S-cysteine at a concentration of 5 μCi/ml. Counts are per follicle; bars represent means ± SEM; and asterisks indicate a significant difference from control group, p < 0.05.

![Figure 5](image2.png)

**Figure 5.** Comparison of minoxidil effects on cysteine and glycine uptake. A set of follicles were incubated for 48 h with both 35S-cysteine (0.5 μCi/ml) and 3H-glycine (0.5 μCi/ml) to compare the effects of minoxidil on these two amino acids. Counts are per follicle; bars represent means ± SEM; and asterisks indicate a significant difference from control group, p < 0.05.
of the dermal papilla, where mitosis normally occurs in anagen follicles [17]. This is in contrast to minoxidil's effects on cultured epithelial cells from both human [18] and mouse epidermis [19]. No mitogenic effects are found in treated human keratinocytes, while in mouse epidermis minoxidil induces a secondary peak of DNA synthesis 3 days after the start of culture. However, both of the keratinocyte studies show that the drug prolongs survival and enhances the growth potential of confluent cultures.

Results of our experiments indicate that minoxidil has direct effects on hair follicles that are unrelated to its main effect of relaxing vascular smooth muscle and thus increasing blood supply to the follicles. The hypothesis that increased blood flow is involved in the drug's mechanism of action is bolstered by the demonstration that topical minoxidil increases cutaneous blood flow in the scalps of bald men [7]. Our experiments do not negate the blood flow hypothesis but do demonstrate that the drug, in vitro, directly increases proliferation and differentiation of cells that form hair shafts.

The organ culture system we describe for mouse vibrissae provides a powerful tool for studying the localization and mechanism of minoxidil effects in follicles. Clearly, vibrissae differ from coat hair and human scalp follicles. Vibrissae are large, provide important sensory input, are surrounded by a thick envelope of both connective tissue and blood sinuses, and have very short periods of catagen and telogen. Even with these differences vibrissae provide a useful model for hair biology. Cultured follicles should also be useful in studying epithelial-mesenchymal interactions and other aspects of the normal biology of hair growth because experiments can be performed in vitro that would otherwise be impossible.
REFERENCES


Figure 9. Minoxidil effects on the autoradiographic localization of 3H thymidine. Follicles were incubated for 8 h with labeled thymidine (2.5 μCi/ml). The arrow indicates representative labeled cells. The control follicle (a) was incubated without minoxidil, while the other follicle was treated with 1 mM minoxidil (b). Bars: 0.05 mm.

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