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# Evaluation of Animal Models Suitable for Hair Research and Regeneration

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## Abstract

Hair loss and regeneration are the subjects of tremendous amount of research for multiple reasons: the well-known importance of hair in individual beauty, the fact that alopecia is a frequent dermatological disease, and that there are limited treatment options. The present work focuses on the evaluation of animal models used for hair research and regeneration. Besides mentioning the option of *in vitro* studies, the chapter analyzes the need of an animal model of alopecia, common used study designs, hair regrowth evaluation methods, and the limitations of the animal models in hair regrowth research. This chapter also discusses the structure of hair, its chemical composition, the properties and functions of hair, consequences of hair loss, the biology of hair loss, and regeneration and existing treatment options for alopecia. By using proper and well thought-out animal models, we aim to refine our knowledge on human hair diseases and hair regrowth. Hair research provides insights into the physiopathological pathways, genetic and cell biochemical mechanisms, and remains a field intensively explored and still inexhaustible.

**Keywords:** animal models, research *in vivo*, hair regrowth, hair regeneration, alopecia

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## 1. Introduction

Hair loss and regeneration is the subject of tremendous amount of research for multiple reasons. First of all, as hair loss or alopecia is a frequent dermatological disease; second, the treatment options are limited and generate variable rates of success. Last but not least, hair is an important component of human outlook with a strong impact on the overall beauty and attraction of an individual. As several studies have shown, hair plays an interesting part in social and sexual communication.

The chapter addresses several issues: the importance of hair from both personal and social perspective, the structure and chemical composition of hair, hair functions and properties, biology of hair loss and hair regrowth, and consequences of hair loss and treatment options. The authors aim to offer an overview of the hair regrowth *in vivo* and *in vitro* studies, focusing on the animal models, and describing the common study designs and their limitations.

The main reason for hair research on animal models relies on the similarities between human and animal skin biology. New treatments for alopecia with different hair growth-promoting agents and various administration techniques have been tested on animal models to prove efficacy and to minimize possible adverse reactions.

## 2. Functions of hair

Also known as “fur” in animals, hair is a defining characteristic of mammals. Besides its important thermoregulatory function, it also has a camouflage purpose and offers protection. In animals, hair follicles can modify their type and density during seasonal coat changes [1]. It is noted that in some species, hair provides sensory and defensive functions, while in others it is used for signaling and communication [2, 3].

Although human hair has lost its main thermoregulatory function, on the scalp, it preserves a heat insulation and cooling purpose, by evaporating sweat from soaked hair [4–6]. It also acts as a sunscreen, offering the skin protection against ultra-violet radiation [7, 8].

## 3. Structure of hair

Hair is defined as an accessory structure of the integument along with the sebaceous glands, sweat glands, and nails [4]. The shaft of the hair (hard filamentous part that extends above the skin surface) consists of three layers, starting from the outside: the cuticle (having several layers of flat, thin cells, overlapping one another), the cortex (containing the keratin bundles in rod-like cell structures), and the medulla (a disorganized and open area at the fiber’s center) [9, 10].

In the dermis, we find the bulb of the hair, which contains the dermal papilla. It has an important role in hair formation, growth, and hair cycle [11]. Besides maintaining stem cells that regrow the hair after it falls out, it also nourishes the hair follicle (providing nutrients and oxygen to epidermal cells in the lower layer) due to the blood vessels present at the bottom of the dermal papilla [1].

## 4. Chemical composition of hair

Hair has a complex chemical structure, containing organic substances (glycogen, acidic polysaccharides, lipids and proteins—amino acids). About 90% of the hair structure consists of proteins, out of which keratin (a combination of 18 amino acids) is the essential component, being produced by the skin keratinocytes. The lipids represent 3% of the hair composition and are supplied by the sebaceous glands or produced in the hair bulb from sterols, fatty acids, and ceramides [12, 13].

Hair also contains inorganic substances (carbon 45.2%, oxygen 28%, hydrogen 6.6%, nitrogen 15%, and sulfur 5.2%) and water. Other mineral components of hair consist of iron, copper, calcium, magnesium, zinc, potassium, and lead, all of them of external sources.

## 5. Properties of hair

The color of hair depends on the type and quantity of melanin inside the cell. The hair follicle pigmentary unit provides the hair shaft color due to the melanin components (eumelanin and pheomelanin) and the interactions between follicular melanocytes, keratinocytes, and fibroblasts (also involved in wound healing) [11, 14]. In the case of the black hair, the pigment is also found in the extracellular compartment.

Hair is flexible and has elastic properties, being able to get longer by 20–50% under controlled traction. Under heat action, the elasticity decreases and hair can break easily. Hair is also hygroscopic, it can absorb water; a fact which decreases hair elasticity and resistance to a third of its normal value [4, 10].

Hair resistance is mostly due to cysteine amino acid, a substance rich in sulphur, which plays an important role in hair cohesion. Hair resistance seems to be increased to physical and biological agents and decreased to chemicals. Excessive light with UV exposure, repetitive hair-dye, and hair perm generate the alteration of the hair elastic properties by the chemical and photochemical degradation of the amino acids from the keratin structure. Hair resistance equals to a force of 60 kg, but it is decreased in children and elderly people. Hair resistance also depends on the hair diameter [4, 6].

## 6. Biology of the hair loss and hair regrowth

Human hair is different from hair grown by mammals due to unsynchronized growth cycles and a sensitive response to androgen.

Human hair exhibits a certain seasonal coordination, but the follicles work independently [15–17]. Latest research results sustain the idea that hair follicles act like neurons, being able to interconnect and generate hair loss and hair regrowth in a small region of the scalp. Human hair has a mosaic pattern as it consists of hair in different stages: the majority of the hair follicles (90%) being in growing phase (also known as anagen), 1–2% in regression (catagen phase), and 8–9% of the hair follicles are resting (in telogen phase) [18, 19]. The cyclic changes from anagen to telogen via catagen involve rapid remodeling of both the epithelial and dermal components of hair follicles [20, 21].

In both humans and animals, hair cycle is influenced by stimulatory and inhibitory factors, such as hormones, growth factors, cytokines, neuropeptides, and pharmaceutical products [18, 22, 23]. The dermal papilla supports an increased cell division and growth rate and induces the shift between anagen, catagen, and telogen [18, 19]. In telogen phase, the old hair is lost, but the follicle will be regenerated in early anagen, when new hair grows up [24, 25].

Current concepts of hair loss pathogenesis include genetic, genomic, hormonal, and immune contributions. Furthermore, the patient's behavior influences the hair density and its strength. In recent years, evidence has suggested that hair loss is a multifactorial disease, and the contributing factors include the resistance to insulin, local pathologies (inflammation, hypoxia, and vascular insufficiency), predisposing physiological factors (menopause and aging), association with other diseases (polycystic ovary syndrome, hirsutism, acne, hormonal imbalances, thyroid pathologies, and other autoimmune diseases). Hair loss remains a consequence of the genotype (hereditary information of the organism)-phenotype (morphology, behavior, and development) interaction [25, 26].

The most common form of hair loss is known as "androgenetic alopecia" (AGA), which represents almost 95%. In this case, hair loss is generated by hair cycle abnormalities, such as the shortening of the anagen, within an abnormal hair cycle, and the anagen-telogen rate shifting from 6:1 to 2:1. Also, hair loss can be due to a small-sized dermal papilla. Both situations lead to shortening of hairs, decreasing hair diameter, shaft loss, and an increased number of hairs in telogen phase. In most situations, the changes of hair diameter (hair thinning) are followed by the loss of pigment: final hair (thick and pigmented) can turn back into vellus (thin and white). Studies point out that another cause of hair loss is the fact that the scalp suffers from vasoconstriction and hypoxia [27, 28].

Hair cycle disturbances are mainly caused by an excess of androgens, which alters the production of regulatory factors (soluble paracrine factors and extracellular matrix components) by the dermal papilla cells [13]. Some specific sites of the body (beard, axillary, and pubic hair) react differently than hair from the scalp, as they are androgen-sensitive [4]. Hair miniaturization and thinning, followed by hair fall is most common in the vertex and the crown-frontal area of the scalp [29, 30].

The occipital part of the scalp is an androgen insensitive area that is why in alopecia, hair is still present in this region, and hair follicles are suitable to be used in hair transplants [31, 32]. The androgen effect on hair can be summarized by the metabolism of the testosterone into 5-alpha-dihydrotestosterone by 5-alpha reductase. A good metabolism limits the hair length (in case of the beard, for example) and deficiencies of the 5-alpha reductase generate enlarged hair diameter (thicker hair in the axillary and pubic area) [19, 33–35].

Another form of alopecia is Alopecia areata (AA), a cell-mediated disease directed against active growing hair follicles. It is a nonscarring alopecia, with limited alopecic patches on the scalp or the body, sometimes affecting also the nails. The pathogenesis of AA includes an autoimmune etiology, linked to human leukocyte antigen (HLA) class II alleles and to the T lymphocytic co-stimulatory cascade [30].

## 7. Genes associated with hair loss

Several studies including recent genome-wide association analyses concluded that a large number of single nucleotide polymorphisms (SNPs) are associated with AGA susceptibility.

So far, only some of the genes involved in hair loss have been discovered: genes AR androgen receptor and EDA2R ectodysplasin A2 receptor from chromosome X, region located at 20p1 on chromosome 20, and additional loci associated with early onset baldness in Europeans, such as *HDAC9* in 7p21.1, *TARDBP* (chr1), *HDAC4* (chr2), *AUTS2* (chr7), *SETBP1* (chr18), q35 (*WNT10A*), chr3q25 (*SUCNR1*), chr5q33.3 (*EBF1*), and chr12p12.1 (*SSPN*) [36].

## 8. Consequences of hair loss

Alopecia can be a part of the normal aging process. Still, hair loss represents a great concern for patients. Several studies have shown that it generates anxiety and distress especially in females, affecting couple and social relationships [37–39].

Hair loss is defined as a stressful experience for both sexes, patients being unable to cope with the progression of the disease [40, 41]. Stress functions not only as a cause, a risk factor, but also as a consequence of hair loss. Alopecia determines a poor quality of life by the physical and psychological sequelae: low self-esteem, depression, distorted social perception, and psychosocial functioning [42–45].

## 9. Hair regrowth treatment

Up to the present, although many treatments have been tested, hair loss continues to be a frequent dermatological condition [46].

Two FDA-approved hair loss treatment drugs: Finasteride (acting on the hormonal cause of alopecia—the excess of androgens) and Minoxidil (acting on the physical cause—the hypoxia due to vasoconstriction), are commonly used in clinical practice in order to treat androgenetic alopecia, which represents 95% of all hair loss causes [38, 47–49]. Minoxidil (1 mg per day) is a topical formulation available in 2 and 5% concentration. It stops hair loss and promotes hair growth as it is a vasodilator and potassium channel opener, allowing more oxygen, blood, and nutrients to reach the follicle [50–53]. It has no therapeutic action on the hormonal and genetic causes of hair loss; therefore, it must be used as a continuous support for the hair follicles, otherwise the hair regrowth will cease and hair loss will begin again in 1–2 months [54–57]. Finasteride is a dihydrotestosterone-suppressing 5-alpha-reductase inhibitor, recommended for male use only, decreasing the serum levels of dihydrotestosterone, stopping hair fall (in 48% of the cases), and stimulating hair regrowth (in 51% of the cases). Studies have shown that 1 mg of finasteride oral treatment has an efficacy similar to daily topical application of minoxidil [58–60]. Given the temporary efficacy of finasteride and minoxidil and the limited number of treatments available in alopecia, new therapies are needed to prevent hair loss and enhance hair regrowth [61, 62].

Pharmaceutical hair loss management also includes different substances (arginine, aminexil, caffeine, and taurine), different peptides, B spectrum vitamins, zinc, or different procedures

(application of stem cells or plasma-rich platelets and low-level laser therapy), even if clinical studies in this respect are lacking. A large variety of over-the-counter products claim to treat hair loss pathology: hair tonics, hair balms, hair masks, shampoos, leave in conditioners, topical solutions, or foams function as potential anti-hair loss agents [43, 44, 63–68].

Alternatives to traditional treatment are laser (low-level laser therapy) and platelet-rich plasma (PRP) injections [47, 69].

## 10. Hair follicle regrowth using gene therapy

Gene therapy aims to deliver genetic material (DNA) into the patients' cells with either a prevention or therapeutic purpose. The therapeutic effect could theoretically be obtained by replacing the mutant gene that causes the disease with a healthy gene, inactivating a mutated gene that causes an imbalance in the organism or introducing a new gene that could fight a particular disease. For the introduction of the gene, a carrier called vector is used, and it usually consists of a modified virus (retrovirus) that will not produce a disease in the organism, but will deliver the gene by integrating the genetic material into the chromosome of a particular cell. The delivery pathway may consist of a direct injection into the tissue or it can be given intravenously, to reach the blood flow [36].

As new evidence shows that 80% of the baldness is genetic, gene therapy could be the solution, although it encounters technical problems that have not been solved up to the present [69]. Most of the hair loss complaints in both female and male patients are due to the presence of androgenetic alopecia, caused by hyperandrogenism and sensitivity to dihydrotestosterone (DHT). It has been noticed that people naturally lacking from birth the 5-alpha reductase enzyme (which converts the testosterone to DHT) never develop androgenetic alopecia [50].

Human scalp has DHT-resistant follicles in the occipital area, this location being used to extract the hair follicles for transplant into the vertex or to the fronto-parietal area [53]. Gene therapy may be a solution in this case, if it can trigger the hair follicles with DHT-sensitive cells and change them into DHT-resistant follicles that could regrow hair without being affected by androgen hormones [70]. Another option would consist of the ribonucleic acid (RNA) interference to block the genes responsible for hair loss. Messenger ribonucleic acid (mRNA) represents the carrier of genetic information from the DNA out of the cell nucleus into the cytoplasm, where it is translated into specific proteins, such as receptors, enzymes. Small fragments of nucleic acids, such as small interfering RNAs (siRNAs), can target a specific gene and block the production of any type of protein in a cell. In hair loss, this technology could be used in order to inhibit the androgen receptor (AR) and the 5-alpha reductase enzymes.

Up to the present, an attempt to effectively control delivery of small interfering RNA using biodegradable cationized gelatin microspheres in an animal model of disease was first performed in 2008. Researchers administered local injections of interleukin-4 and neutralizing anti-interferon- $\gamma$  antibody in C3H/HeJ mice. They concluded that alopecia areata was effectively treated as the treatment suppressed CD8 T cell infiltrates around the hair follicles and

repressed enhanced interferon- $\gamma$  mRNA expression in alopecic skin. Also, restoration of hair shaft elongation occurred due to Th1 transcription factor T-box 21 small interfering RNAs conjugated to cationized gelatin [71]. Another recent study showed that the sonic hedgehog (*shh*) gene stimulated the hair shaft production and anagen phase in C57BL/6 mice, after being delivered with an adenovirus vector [72].

Gene therapy is currently available only in research settings. It represents a promising therapeutic option for several diseases (especially those with no cure for the moment), but this procedure needs more research and improvement that need to be considered safe and to prove its effectiveness. So far, scientists have encountered difficulties in finding proper delivery pathways of the genes to the body, targeting them to particular cells, controlling the new gene(s) and their effect after they have been inserted into the body [73].

## 11. Hair regrowth studies *in vitro*

Human hair follicles as research material for hair loss and regeneration involve ethical problems, an invasive collection method and a limited quantity of follicles available for extraction and testing [60, 61].

The first methods of isolation and maintenance of hair follicles in cell cultures go back to 1990, when several researchers used this method in order to study the biology of the hair cycle [74, 75]. Follicles were usually taken during face lifting surgery, but only a third were suitable for the isolation phase of the hair transplant, due to improper collection procedures. The follicles needed to be isolated from human scalp in a few hours, maintained at 2–6°C, in an Earl medium, combined with phosphate buffered saline solution, with calcium and magnesium added. Only the follicles that seemed intact were used.

*In vitro* hair research was supported by the identification of growth factor function in the process of hair regrowth and differentiation [76–79]. Philpott et al. have reported that in the absence of insulin, follicles prematurely enter the catagen stage [80]. Subsequent *in vitro* and *in vivo* studies, in murine and human models of hair follicles, have demonstrated that IGF-1 level is a regulation factor of hair growth and together with IGF-1 receptor influence hair growth cycle.

Other studies performed in 1990 have shown that transforming growth factor beta 2 (TGF- $\beta$ 2) promotes anagen to catagen transition. Several inhibitors of hair follicle growth *in vitro* have been identified such as interleukins (IL-1 alpha and beta) and tumor necrosis factor (TNF-alpha). Researchers concluded that these cytokines play a significant part in the pathophysiology of hair inflammatory diseases. Although the factors that perform the transition *in vitro* from anagen to catagen have been discovered, inducing a full hair growth cycle has not been made possible yet. Murine models of hair follicles, isolated at different growth stages *in vitro* seem to maintain their cyclic activity and to illustrate their status *in vivo* [81].

On the other hand, healthy human dermal papilla cells, isolated from hair follicle, lose the ability to produce hair growth when being outside the body. Also, cycling hair follicles cannot be maintained in culture for any length of time [82].

The data that we now possess about the life and function of the hair follicle in health and disease rely on the successful research performed *in vivo* (experiments on natural animals and genetically manipulated models) and *in vitro* (cultures of a cell type—dermal papilla or organ culture of isolated cell follicles). The preference for one of the two experimental alternatives depends on several factors: the purpose of the research and the advantages and disadvantages involved.

## 12. Hair regrowth studies *in vivo*

### 12.1. The need of animal models

Animals and humans are remarkably similar at physiological and anatomical levels. Also, genetically speaking, we share 67% of our DNA with earthworms and 99% with mice. Almost 90% of the veterinary medicines used to treat animals are similar to the ones developed for human use. Animal models can mimic human responses, but the differences in species and even in individual animals must be taken into consideration [83]. By recreating human diseases in animal models, we can study and understand the physiopathological processes involved in the disease and maybe find an efficient cure. The first Nobel Prize was awarded in 1901 and other 94 prizes were directly dependent on animal research [84].

Laboratory animals are used when human testing is not available for practical or ethical reasons. Animals represent good research subjects as they have a shorter life cycle that enables scientists to observe the animal throughout the entire life and across several generations. Also, animal models can be easily influenced by the environment, which is controlled by the researcher as far as the diet, temperature, lighting, and other factors are concerned.

Researchers use animal models for short-term objectives (to determine how the animal model responds to a stimuli or a treatment) and long-term purposes (development of a new drug, evaluation of bioavailability or toxicity, genetic study). The animal model should be sensitive, appropriate for the studied condition either by using specific evidence of previous studies or using a new animal model with the risk of generating inaccurate results [69]. Besides the similarity with the human response, other key features of the biomedical research on animal models are specificity to the study purpose, validation of the animal model, and improvement for further research. Animal research has brought many benefits not only to humans but also to animals in disease prevention and treatment [47, 48, 69].

For more than a 100 years, almost all the information obtained in the human and animal health research has been the result of studies performed on animal models. The most common aim of animal models use is the development of new methods for the diagnosis and treatment of diseases, through an understanding of the biology and the physiopathological processes involved [47, 69].

Even though animal models remain a necessity, alternatives consist of computer models, tissue and cell cultures, and other nonanimal-related research methods. In order to minimize the

harmful effect of research performed on animal models, scientists tend to reduce the number of animals used to obtain valid results, to refine the experimental technique, or replace it with nonanimal research methods.

## 12.2. Animal models used in hair loss and regrowth

A large variety of animals (mice, rats, hamsters, rabbits, sheep, and even stump-tailed macaque) provide useful models for the *in vivo* study of hair loss and regrowth, but 95% of the animals bred for research purposes are rats and mice [85–89].

Mice represent an excellent model to study the hair cycle for several reasons: the first two cycles of the mouse hair follicle are synchronized; the mouse hair cycle is short, lasting for 3 weeks; hair follicles can be easily harvested and examined at specific time points in the cycle. Most importantly, the stages of the hair cycle have been well characterized in the mouse: anagen being morphologically subdivided into six stages and catagen into eight [22]. The periodic intervals of rodent hair cycles (especially the anagen-growing phase) seem to be less susceptible to iatrogenic influences [90]. The mouse hair cycle does not differ structurally from the human hair follicle cycle, except for the fact that during catagen the hair bulb is remodeled, but the vibrissae follicles do not retract. Scientists have recently discovered that a certain progenitor cell population in mice is analogous to the human cells, encouraging research on this particular animal model.

Besides studying the normal hair cycle on mice, scientists also focussed on the growth waves and hormonal control [91]. Significant differences between species regarding the follicular function and limited androgen-sensitive models were noticed [92]. Spontaneous mutations have been discovered and studied on hairless, nude, and tabby mutants, waved and angora animals, leading to the identification of new genes involved in hair loss and opening the path for transgenic technology research [93, 94].

Transgenic mice, also known as “knockout mice,” are mice with altered genome through the use of genetic engineering. This gene-targeting technique has revolutionized the biomedical research by offering researchers the ability to create a specific animal model for the most common human diseases. In order to select the most appropriate immunodeficient mouse models for research purpose, scientists also take into consideration: background strain, behavior, husbandry, disease susceptibility, life span, breeding performance, radiosensitivity, functionality of various endogenous immune system components, and leakiness (tendency to produce functional B and T cells as they age).

Up to the present, immunodeficient mice (with T and B cells deficiencies) were used as models for autoimmune disease mechanisms and androgenetic alopecia studies. The androgen action upon the hair follicles has been studied on spontaneous and genetically engineered nude mutant mice [95].

The C57BL/6 mouse is the most popular laboratory rodent, widely used and studied, having its entire genome published. Research applications using this particular type of mice include immunology, cancer, neurodegenerative disease, age-related hearing loss, bone density, diabetes,

obesity, and biomarker studies. This black coat mouse has been used for the skin-free pigment and early visible pigmented tips of new anagen regrowth [88]. C57BL/6 represents one of the most well-characterized models available, with a minimum risk of genetic drift. It is also a convenient model for creating transgenic mice, which are recognized by the mixed coat colors.

The C3H/HeJ mouse model was used in a large range of studies: immunology, cancer (especially mammary tumors), inflammation, sensorineural, and cardiovascular disease. This animal model was the most widely reported for hair growth promotion, most possibly due to the fact that C3H/HeJ mice can spontaneously develop alopecia areata (AA) from 6 to 18 months of age. Also, alopecia areata can be surgically induced by skin-grafting from a donor animal with AA onto an isogenic C3H/HeJ recipient (normal haired mice of the same strain) [90, 96].

In 2010, researchers created the first rodent model of AGA, taking into consideration its relationship to androgen metabolism and androgen signaling, mediated by the androgen receptor (AR). They used transgenic mice overexpressing human AR in the skin under control of the keratin 5 promoter and exposed them to high levels of 5-alpha dihydrotestosterone, which led to delayed hair regeneration, mimicking AGA. The scientists concluded that androgen-mediated hair loss is AR-dependent and suggested that AR and beta-catenin mediate this effect [97].

There are many rat strains raised for research purposes, but the albino Wistar Bratislava rat is the most commonly used. Gene knockout techniques are relatively difficult to be applied and successfully achieved in rats. For hair loss and regeneration experiments, the Wistar rats and the Dundee Experimental Bald Rat (DEBR) strain were commonly used. The latter has the ability to spontaneously develop adult onset alopecia areata (AA) at a higher frequency than in the mouse model [98].

In the research field of hair loss and regeneration two major achievements must be mentioned on the rat animal model: coaxing human stem cells to become dermal papilla and producing new hair follicles when transplanted on rat skin [98]. Also, by inhibiting the rejection of foreign skin, human skin grafts were applied and even rat dermal papillae continued to produce hair after reimplantation *in vivo* on a rat model [99, 100].

Research performed on a rabbit animal model, added important data to the field, proving that full thickness transplants, made with full pedicle graft (separated from their original nervous and vascular supply) retain their original *intrinsic* activity and are not modified by the action of the surrounding tissue [101]. Furthermore, the rabbit represents a common animal model used to screen compounds potentially efficient in treating alopecia.

The Golden Syrian hamster (*Mesocricetus auratus*) has been previously used for research purposes, even though it is a very common pet. The hamster flank organ has served as a model to study the effect of testosterone (T) upon the hair follicle, the sebaceous glands, and the dermal pigment. This hamster is known to be useful for the specific and quantitative assessment of different substances on hair growth, being also useful for therapy testing in hirsutism. Macroscopic (hair density evaluation) and microscopic (hair diameter analysis) hair growth assessments have been performed on Golden hamsters [102].

### 12.3. Common study designs in hair loss and regrowth

#### 12.3.1. Housing conditions

*In vivo* hair regrowth studies usually use animals of either sex and weight, kept in experimental rooms that are free of pathogens and opportunistic agents. For 7–14 days prior to the experiment, the animals are housed under specific conditions: room temperature of 23°C, controlled humidity, a 12:12 h light, and dark cycle. In order to avoid licking, individual housing is preferred or a maximum of two animals per cage. Standard laboratory diet and water ad libitum are provided. After completing the experiment, animals are euthanized according to the current regulations. For accurate results, most of the studies on animal models are performed in triplicate [47, 69].

#### 12.3.2. Depilation methods

Experimental designs may include one of the depilation methods: shaving, the use of a raisin mixture, or a hair removal cream [91, 103]. The most commonly used is the shaving of a larger skin area (the whole back or body) or of several smaller areas that are denuded for testing. For animal immobilization during procedures, general anesthesia is commonly performed with a combination of ketamine (i.p. 50 mg/kg b.w.) and xylazine (20 mg/kg b.w.) [47, 48, 69].

Some study designs, such as that of Mester et al., required before each successive hair treatment, the shaving of the skin. This procedure can induce mechanic stimulation of hair growth, as previously reported in the literature, and influence the study results. Other experiments done on adult rats point out that after the fur was dyed and shaved, the regrowing hairs formed a system of linear loops that were closely correlated with the shaving process [66, 67].

In order to avoid this effect, it is recommended not to shave the skin of the animal model before each session of therapy. Other factors which influence the hair regrowth are physical factors such as low temperature, which triggers fast regrowth after shaving.

Depilation-induced hair cycle has been studied, and it follows a strict course: nine days after depilation, the hair follicles enter the final stage of the growth cycle (anagen VI). On day 17 after depilation, the follicles enter the regression stage (catagen), while on day 20 follicles get to the resting stage (telogen) [22].

#### 12.3.3. Evaluation of hair loss and hair regrowth

Efficacy of the treatment is screened by observing the presence, rate, and cosmetic acceptability of hair regrowth. More sophisticated assays include determining how the drug induced hair regrowth and exploring the pathogenesis of AA.

Researchers do not possess standardized methods for *in vivo* hair regrowth assessment. New, accurate, and minimally invasive procedures are still needed as the most commonly used tools are qualitative assessments, limited in number. They include macroscopic assessment with the naked eye (visualization and photographs of the area of interest) based on scales that assess the percentage of hair regrowth on the interest area and tricoscopic evaluation (with a

hand-held dermatoscope, with polarized light and magnification abilities) [41]. Trichoscopy allows a correct hair regrowth evaluation, as it can detect decrease of hair diameter up to ten times or diameter variations. Both macroscopic and microscopic methods assess hair regrowth with the help of personalized hair growth scales or standardized, already published scales.

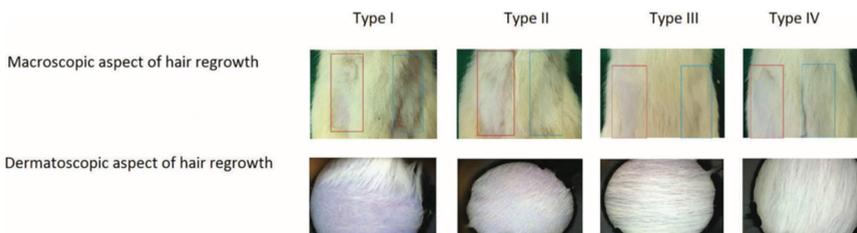
Usually, the dorsal part of the animals is used for the testing. After being depilated and treated, the animal skin is observed and photographed at specific time intervals (day 1, 7, 14, and 21) to record the start of the hair regrowth period and the pattern of hair regrowth, compared to controls. Several hair regrowth potential scores are mentioned by literature. The one described by Matsuda et al., for instance, ranges from 0 to 5: 0 = no hair growth, 1 = less than 20% of hair growth, 2 = 20–39% of hair growth, 3 = 40–59% of hair growth, 4 = 60–79% of hair growth, 5 = 80–100% of hair regrowth [54]. Researchers also use self-designed scales of hair regrowth that consider: Type IV (high hair density, full, thick fur), Type III (moderate hair density with no visible skin area), Type II (low hair density, with the visualization of the skin), Type I (uneven hair growth on the test area, skin easily seen) [47, 69].

The hair regrowth potential scores can be applied for both macroscopic and microscopic assessments (**Figures 1 and 2**).

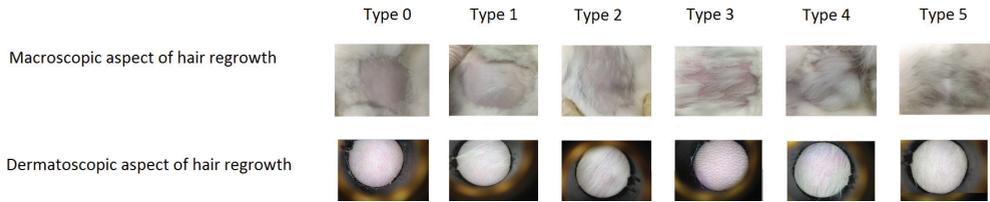
On the other hand, quantitative methods, such as hair weight determinations, hair density measurements, or histopathological examination offer more accurate results. For hair weight determination, the regrown hair from an area of 1 cm<sup>2</sup> of skin is cut and weighed with an analytical balance [48].

In order to analyze the histological features at the end of the treatment period, the animals are sacrificed and a skin biopsy is isolated for histopathological examination. The thickness of the skin and the location of hair follicles in the dermis can be assessed by microscopic photography.

Also the hair cycle can be assessed, as the anagen induction can be calculated with the formula:  $(\text{number of follicles in hypodermis}) \times 100 / (\text{number of follicles in dermis})$ . Literature data showed an association of increasing skin thickness, follicle count, and macroscopic development of skin pigmentation with anagen induction [18, 23]. The study by Liu et al. found that in the anagen phase the bulb of the hair follicles was enlarged and deeply inserted into the dermis. The research also revealed that the hair follicles in the shaved, bare areas were short, small, and in the phases of telogen, anagen, or catagen [69].



**Figure 1.** Classification of the hair regrowth effect (type I, type II, type III, type IV) for macroscopic and microscopic assessments—personal study performed on Wistar Bratislava rats. The control area is marked with red (left side of the picture), the test area with blue (right side).



**Figure 2.** Classification of the hair regrowth effect (type 0, type 1, type 2, type 3, type 4, type 5) for macroscopic and microscopic assessments—personal study performed on New Zealand Rabbits.

The hair growth cycle, consisting of three phases (anagen, catagen, and telogen) is used by both practitioners and researchers to diagnose the hair growth condition and to decide on the hair growth-promoting agent. In human subjects, digital trichoscopy is available, with automatic assessment of the number of follicles in each hair growth phase.

Several studies focused on the validation of Minoxidil 2% treatment on the animal model used, as this topical treatment is thought to be the gold standard treatment for hair loss. This substance affected the normal hair cycle by shortening telogen, causing premature entry of the resting follicles into anagen phase [103, 104].

#### 12.4. Limitations of animal models regarding hair regrowth

The limitation of using an animal model while studying hair regeneration can be briefly summarized as follows. First, synchronized hair cycles generate waves of new hair regrowth, which make the interpretation of result a hard task. Second, the lack of independence of the hair follicles, since they have coordinated regrowth pattern on a precise time scale, as described by Muller-Rover et al. [22]. Third, young mice present the drawback of patchy growth after the second wave of hair growth is completed. Lastly, the increased hair density on an animal model leads to difficulties of assessment by densitometry or cross-section trichometer [69].

The results of our hair growth research performed on Wistar rats showed, besides a normal hair growth in the majority of the animal models, a lack of hair regrowth on the tested area. Other studies performed on black-and-white mice reported that no further hair growth was observed on half of the control animals.

We also noticed a diffuse hair regrowth in some study groups, while in others, the hair appeared to make some specific linear loops that were observed macroscopically. Literature date confirmed our findings. In similar situations, researchers experienced a diffuse hair growth in some animals with an uncharacteristic, diagonal strip [66]. Li-Yaun Liu et al. described four main linear hair regrowth patterns noticed on a rat model: the dorsal loop and the lateral dorsal loop (running along the dorsum and hind limb) and the ventral loop and lateral ventral loop (traveling along the thorax, abdomen, and forelimb). These hair-loop-lines create cranio-caudally-oriented waves of regrowth 2–15 mm wide, symmetrically on both sides of the body, running from the head through the torso to the limbs [105]. Li-Yaun Liu et al. concluded that after shaving the skin, the hair follicles from these new hair lines were always in an anagen phase [106].

Also, the behavior of the animals should be taken into consideration, as it can create issues and interfere with the research results [107]. For example, the C57BL/6 mice show barbering behavior, the dominant mouse in a cage selectively removing hair from its subordinate cage mates. Mice that have been barbered have large bald patches on their bodies, especially around the head, snout, and shoulders [108].

Regardless of the shortcomings of either animal model, most of them validate their usefulness for drug efficacy and safety testing for humans.

### 13. Conclusion

Although the studies performed on animal models encounter both technical and objective issues, further scientific research is not impeded and continues to remain an intensively explored field. By using proper and well-thought out animal models, we aim to refine our knowledge on human hair diseases and hair regrowth. Hair research provides further insights into the physiopathological pathways and genetic and cell biochemical mechanisms that could promise the cure of hair loss.

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