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75 Animal Models

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Introduction

Skin aging is influenced by a combination of biological, physiological, and environmental factors. While in vitro models and bioengineering techniques can reproduce one or more of these factors, they cannot encompass the numerous components existing in living tissue. Animal models mimic the combination of influences contributing to skin aging in humans. Though many potential animal models exist, studies on skin aging use relatively few lab species. The following describes some of the experiments that utilized animal models. They are divided into two categories, actinic and intrinsic aging experiments. Hopefully, it will serve as a starting point and resource for those wishing to develop further knowledge and interventions.

Actinic Aging

Most experiments utilize rodentia because they possess the characteristics needed for studies on skin aging. Mice and rats in particular are useful for numerous reasons. First, genes in mouse and man are >99% conserved. In addition, the ability to add to and selectively alter the mouse genome increases opportunities for using the mouse to understand the genetic basis of human health and disease [1]. Therefore, the mouse provides a good model for many human diseases, including skin disorders.

The first attempt to observe connective tissue damage via UV radiation utilized the Dublin Imprinting Control Region (ICR) Albino Random Bred mouse. The albino mouse, a laboratory strain of the house mouse, Mus musculus, is characterized by a mutation in the *Tyr* locus on chromosome 7, which codes for tyrosinase, an enzyme important for the proper production of melanin. In 1964, Sams et al. shaved the backs of mice and subjected them to long-term exposure with UV, which produced the first report of observed elastosis on the dorsal backs of the animals [2]. The study did not differentiate between results of UVA and UVB exposure but instead recreated the effects of long-term exposure to sunlight in humans, a feat that had failed up to this point in other animal models, such as the guinea pig. However, the study used doses of UV radiation exceeding those normally experienced by humans, and the tumors developed by the mice were thought different from those produced in man. Thus, scientists concluded that the albino mouse was not a suitable model for elastosis and turned to the hairless mouse.

Due to events mentioned above and a report by Winkelmann et al., which discussed another experiment that produced the same tumors in hairless mice as those seen in humans, hairless mice became the animal of choice [3]. Hairless mice possess mutant alleles at the *hairless (hr)* gene, which lies at the 70 Mb position of chromosome 14 [4]. The strains have proven valuable in various photobiologic investigations ranging from phototoxicity, photoimmune effects, carcinogenesis, and UV-induced DNA damage [5]. In addition, the UV-induced changes are comparable to those in human skin and are therefore very relevant. The action spectrum for the acute responses to UV radiation edema in the hairless mouse is comparable to that for sunburn erythema in humans, and the time courses for both responses are similar [6]. Further, the UV-induced connective tissue damage that occurs is largely analogous to that in man [7]. These mice occur in two varieties, albino and pigmented [1], and the option of pigment can add another dimension to experimental possibilities. However, the most commonly used hairless mouse for photoaging is the albino Skh-hairless [8].

Elastosis has been the most common method for assessing damage caused by long-term UV exposure. The first study designed to produce elastosis in a hairless mouse was performed by Berger et al. in 1980 using the naked (Ng/–) albino strain [9]. Since then, numerous experiments have recreated elastosis from exposure to ultraviolet radiation. Johnston et al. exposed hairless mice either to UVA or UVB radiation on alternate days to assess the consequences of long-term exposure on the connective tissues of the skin. Hydroxyproline and desmosine were used to interpret collagen and elastin concentrations. Desmosine content of the skin, which was used as an index of cross-linked elastin, was increased in mice treated with UVA or UVB. In contrast, collagen content, measured as hydroxyproline, was the same in all

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treatment groups [10]. Despite the similarities in collagen content, the experiment demonstrated through biological assays that collagen synthesis via prolylhydroxylase (PH) was impaired with UVA exposure and thus may lead to decreases in collagen synthesis with increased time and dermal atrophy. Studies on elastosis caused by UV radiation continued with histochemical studies by Kligman et al. [7], and with electron microscopy, biochemical and immunochemical experiments [11].

The former studies emphasized the effects of UV light on elasticity, a dermal component. Some studies demonstrated the effects of photodamage on the viscosity of the skin, an epidermal component. Fujimura et al. treated female HR/ICR hairless mice topically with 1,25-dihydroxyvitamin D_3 to assess its effect and contribution to photo wrinkling [12]. 1,25-dihydroxyvitamin D_3, or 1,25-dihydroxycholecalciferol, is the active form of the vitamin produced in the kidneys. In humans, it is made from 25-OH-cholecalciferol, which arises from cholecalciferol in the liver. Cholecalciferol is obtained directly from diet but can also be manufactured in the skin from 7-dehydrocholesterol. This conversion occurs in the epidermis and requires ultraviolet light.

The mice were treated once daily, five days per week with 1.00 µg, 0.20 µg, or 0.05 µg of 1, 25-dihydroxyvitamin D_3. Skin sagging was assessed using a scale described by Bisset et al. [13] four grades were used, with the fourth being the most severe. After six weeks of treatment with 1.0 µg/day of 1,25-dihydroxyvitamin D_3, skin appearance deteriorated, as there were coarse, deep wrinkles across the backs of the mice.

1,25-dihydroxyvitamin D_3 also had an effect on the mechanical properties of the skin. U_e or immediate distention, a factor measuring elasticity, did not change after topical administration. However, U_v or delayed distention, a parameter of skin viscosity, increased remarkably after 1,25-dihydroxyvitamin D_3 application at all doses. Note that U_e is largely a dermal component, whereas U_v is considered epidermal. This study demonstrated the effects of high levels of 1,25-dihydroxyvitamin D_3 on the viscosity of the skin and suggested that changes in the mechanical properties after topical $1,25(OH)_2$ VD_3, treatment are due to physical changes in the epidermis [12].

The experiment also measured two other parameters, U_f and U_r. U_r represents immediate retraction, whereas U_f indicates final distention. The ratio of the two, U_r/U_f, may be used as a general parameter of aging. Usually, the ratio decreases with age-related changes due to a decrease in U_r. However, in the study by Fujimara et al. the decrease in the ratio was due to an increase in U_f, which suggests a distinction from normal age-related changes in the skin. Further studies may later reveal reasons behind these discrepancies.

In addition, higher dosages of vitamin D have been recommended for prevention of osteoporosis and increased calcium absorption. However, this study suggests that high concentrations of vitamin D may contribute to symptoms in other areas of the body. Future experiments may want to explore the balance between benefits of taking vitamin D and the risks of accelerated degradation in other systems.

The degradation of collagen and other components of the dermal extracellular matrix can partially be explained by the upregulation of matrix metalloproteinases (MMPs). Matrix metalloproteinases, a family of nine or more zinc-dependent endopeptidases, cleave the constituents of the extracellular matrix in connective tissues [14]. The enzymes have also been implicated in other pathologies such as atherosclerosis and emphysema. Their association with human aging has been revealed in studies showing the upregulation of matrix metalloproteinases in cultured fibroblasts after irradiation [15]. Ultraviolet (UV) B induces expression of MMP-1, MMP-3, and MMP-9, whereas UVA induces expression of MMP-1, MMP-2 and MMP-3 [16, 17]. Saarialho-Kere et al. ascertained changes in matrix metalloelastase (MME) in hairless mice skin using immunohistology. Anti-MME antibodies detected presence of the enzyme in samples taken after repeated irradiation. The first sample, taken after 12 days, indicated the dermis as the location of most MME. The last samples at 8 and 11 weeks showed a strong signal for the subsistence of MME. In every specimen, stromal cells in the dermis resembling macrophages or fibroblasts contained the enzyme, but no epidermal cells showed MME signals.

The application of medications to the reversal of skin aging has also been studied using the hairless mouse. *Lorraine Kligman* was one of the first to illustrate the restorative capabilities of topical all-*trans*-retinoic acid (RA). Skh *hairless*-1 albino mice were irradiated dorsally thrice weekly for 10 weeks to produce the damage. She then used three groups of mice receiving RA in various percentages or different vehicles to analyze the effects. The skin was studied histologically and with electron microscopy [18]. Via these methods, she found evidence of repair to UV-damaged dermis of mouse skin and illustrated the repair was a direct result of increased collagen synthesis by hyperactive fibroblasts stimulated by RA. These experiments helped to establish the use of topical retinoids for repair of photodamaged skin.

An innovative model for future regenerative processes is described by Byung-Soon Park et al. Their purpose was to assess the potential for using

adipose-derived stem cells (ADSCs) in treatments for aged skin. ADSCs have previously been studied for use in wound repair, and experiments have illustrated their ability to stimulate collagen synthesis and migration of fibroblasts [19]. Since the damage resulting from UV exposure includes degradation of collagen and deceleration of collagen synthesis, ADSCs were postulated a potential cosmetic treatment for photodamage. To find out, ADSCs and conditioned media of ADSCs (ADSC-CM) were injected intradermally on the backs of three micropigs, twice in a 14-day interval. One month after the second injection, skin samples from the injection site were evaluated histologically and by Western blot. Western blot study showed a remarkable increase in collagen, supporting the possibility for future use of ADSCs and their secretory factors in the treatment of skin aging [19].

UV radiation is included in a category of skin insults known as oxidative species. Alcohol consumption, ozone exposure, and cigarette smoking are included in this group, and tobacco smoke has an especially deleterious effect on the extracellular matrix of the skin. This is demonstrated in in vitro studies harvesting fibroblasts treated with tobacco smoke extract [20]. There is evidence from in vivo studies that tobacco smoke induces premature skin aging [21]. The first study examining changes in the connective tissue matrix of hairless mice due to cigarette smoke was performed in 2007 by Tanaka et al. Aqueous smoke solution was prepared using the smoke from cigarettes dissolved in phosphate buffered saline. This extract was topically or intracutaneously administered to the backs of male hairless mice thrice weekly for six months.

After six months, skin specimens were taken and stained with hematoxylin and eosin (H&E). A monoclonal anti-collagen type I antibody was added to better identify collagen bundles. In the treated sample, there was a loss of discernable collagen bundles in the dermis when compared with the control. This study provided the first direct evidence that tobacco smoke induces premature skin aging using an in vivo method [21]. Leow and Maibach summarized the studies that had used Laser Doppler Flowmetry (LDF) and other methods to measure the skin's blood flow. Despite differing methods, instruments, and test populations, there was consistent decrease in cutaneous blood flow during the first 2 minutes of smoking cigarettes [22]. No changes were found in nonsmokers. A later study by Manfrecola et al. observed a 38.1% reduction in smokers and 28.1% reduction in nonsmokers, but the recovery time was less for nonsmokers than for smokers [23]. Both studies suggest that decreased blood flow may be a contributing factor to visible signs of skin aging. However, additional studies on the effects of cigarette smoke in animal models would provide further molecular evidence and corroboration.

Intrinsic Aging

The literature addressing innate aging is not as cohesive as that for actinic. This is because the study of intrinsic aging is more difficult for a few reasons. First, the consequences due to one measurable factor, such as UV radiation, can be observed more quickly than those from biological or physiological influences. Actinic aging describes an accelerated process. The skin's degeneration is related to the amount and length of exposure, and the results are visible. Intrinsic aging in humans occurs over years, and it becomes more difficult to assess the effects that biological or physiological components have on the skin, free of environmental influences. In addition, scientists have yet to prove the exact reasons for the different rates of aging among individuals. Therefore, the experiments mentioned below are the beginning to the further understanding some of the processes involved with intrinsic aging using animals.

Hiromi Kimoto-Nira et al. discussed the use of senescence-accelerated mice (SAM) to observe various physical changes associated with aging, including those of the skin. Senescence-accelerated mice develop normally, but then show an early onset of aging and allow scientists to observe processes that may be similar in humans over a shorter length of time. This particular experiment described the process in terms of bone density loss, incidence of skin ulcers and hair loss. *Lactococcus lactic* subsp. *cremoris* H61(strain H61), a probiotic, was administered to a specific substrain of senescence-accelerated mice, SAMP6, orally for 5–9 months [24]. The result was decreased incidence of skin ulcers and a lesser degree of hair loss, both assessed using a grading score system developed by Hosokawa et al. in 1984 [25].

In 1975, a study addressed changes in connective tissue on a more molecular level and helped to illuminate the relationship between hydroxylysine-linked carbohydrate units in collagen molecules and age. Murai et al. used the fact that glycosylation of collagen renders the molecule more insoluble to separate collagens obtained from young and old rats into soluble and insoluble fractions. They then sampled the insoluble fraction and determined the extent of glycosylation. They observed a decrease in glycosylation of hydroxylysine in collagen during maturation followed by a gradual increase in proportion to age [24]. Note that at the time of the experiment, the role of glycosylation in the formation of collagen molecules was

not understood. Yet, the experiment utilized an animal model to observe molecular differences in collagen due to age. The study also suggested that the changes observed in skin collagens could be extended to collagens in other connective tissues.

Though rats have not been examined as closely as mice genetically, they are frequently used in studies concentrated on the skin. The Ishibashi (IS) rat, a cross between Wistar and wild rats, has a unique skin appearance characterized by wrinkles and furrows appearing at an age of 12 weeks. Although the rats demonstrate the physical symptoms associated with actinic aging, the changes occur independently from the influence of UV radiation. Sakuraoka et al. explained the wrinkles by analyzing elastin and collagen content in young and old IS rats [26]. As a control, they assessed the same factors in young and old Sprague–Dawley (SD) rats. Collagen content was determined by measuring hydroxyproline content in back skins according to the method of *Prockop* [27]. The dermis was blended with 0.5 M acetic acid, digested with pepsin (a proteinase) at 4°C for several hours, and centrifuged. The resulting supernatant was dialyzed against 0.02 M dibasic sodium phosphate in order to precipitate collagen. This precipitate was then subjected to 5% SDS-PAGE containing 3.6 M urea under non-reducing conditions. Coommasie brilliant blue (CBB)-R250 was used to stain the gels, and the bands were examined using a densitometer. The elastin content in IS rat skin was estimated using isodesmosine, an isomer of cross-linking structures composed of four elastin molecules. The isodesmosine content was obtained by hydrolyzing the skin samples with 6 M HCl for 18 h at 110°C and by using high performance liquid chromatography. No significant differences in skin collagen content were discovered between matured and aged tissues in either IS rats or SD rats, which suggested collagen may not be related to the signs of aging seen in the IS rat. The findings were different with respect to elastin content, and the aged IS rat skins had significantly less isodesmosine than the skins from their younger counterparts. The notable reduction of isodesmosine in the aged IS rat skins and lack of isodesmosine changes in SD rat skins suggest that isodesmosine content may be related to the observable cutaneous aging of IS rat skin [26]. The authors proposed that the wrinkling occurring in IS rats is due to the decrease in elastin content and may be a good model for the study of skin aging in humans.

Although methodologies for experiments on skin aging have been dominated by the use of rodentia, there have been other mammals that have also been effective. Hairless dogs have been used to investigate the improvement of aged skin after topical treatment with kinetin. The dogs were descendants of Mexican hairless dogs that showed age-related changes in their skin structure. Their epidermis had spotty pigmentation, and lesions had heavy deposition of melanin granules. The left dorsum of each dog was treated with kinetin (KN) solutions while the right side served as the control. After about 50 days of topical application, the KN-treated sites showed normalization of hyperpigmentation and skin rejuvenation. In addition, there were no harmful effects on the skin of the dogs, which suggests this may be a safe long-term treatment for humans [28].

FGF23 and Klotho as Future Models

Fibroblast growth factor 23 (FGF-23) null mice and *Klotho* mice are two transgenic strains with phenotypes resembling human premature aging, such as short lifespan, arteriosclerosis, osteopenia, ectopic calcification in various soft tissues, pulmonary emphysema, impaired maturation of sexual organs, senile atrophy of the skin, and defective hearing [29]. They have been used as models in experiments studying the effects of aging, particularly those correlating the levels of vitamin D with aging processes. As suggested by the experiment of *Fujimara* et al., high levels of vitamin D_3 are correlated with aging skin. The premature aging symptoms in *Klotho* and *FGF-23* null mice are related to high serum levels of phosphate and increases in vitamin D activity [30]. The similarities observed between the two strains have led to the discovery that the proteins encoded by the mutant genes are linked via a common pathway; in fact, the FGF-23 protein is unable to induce renal phosphate wasting in the absence of the Klotho protein [30].

Conclusion

The examples presented are but a few of the existing investigations of skin aging. The representation of all related studies would require an entire textbook. In addition, these studies only address animal models, and no discussion of those involving human models, xenografting, or bioengineering methods has been included. It is hoped that this text will serve as a beginning to a more complete acquaintance with the methods used for the study of human skin aging.

Though many animal models have been described, there is room for development. As the understanding of the aging mechanism becomes more intricate, new models with processes resembling those in man will be needed

to further knowledge of human risk. Further, many of the experiments described above observe aging processes in the skin outside of any influence from age-related pathologies. The *Klotho and FGF-23* mice are good models for illustrating problems simultaneously associated with systemic and skin aging. Perhaps in the future, similar models will identify the relationships between systemic processes and physical appearance. Future cosmetic advances may then lie in treatment of the entire body instead of localized skin corruption. Hopefully, as the readily accessible skin permits aging amelioration, the information will lead to advances with other organs.

Cross-references

Basophilic (Actinic) Degeneration of the Dermis: An Easy Histological Scoring Approach in Dermal Photo-aging

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