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The effect of aging on skin cells in domestic rabbits, a histological study using a special dye

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Abstract

The study provides a comprehensive analysis of the histological changes in the skin of domestic rabbits with age using Verhoeff for collagen stain, which is considered a special dye. A group of histological staining techniques was used to examine skin samples taken from rabbits at different age stages. We were able to analyze and identify changes in the structure of the skin, including the dermis, epidermis, and subcutaneous fatty tissue. The results showed that aging leads to clear changes in the structure of the skin, including an increase in the thickness of the dermis and epidermis, a change in the distribution of collagen and elastic fibers, and the appearance of fatty aggregates. An increase in inflammatory cell activity in the skin has also been observed with age, indicating an effect of age on the skin inflammatory response. In addition, the study revealed changes in the composition of blood vessels in the skin, as an increase in the size and number of blood vessels was observed in aging skin tissue. These changes reflect the effects of aging on skin circulation, which may affect skin tissue nutrition and its ability to regenerate.

This study provides a comprehensive understanding of the histological and functional changes in the skin of domestic rabbits with age. The results indicate that aging significantly affects skin structure and function, reinforcing the need to develop new therapeutic strategies to maintain skin health and delay signs of aging.

Keywords: Aging on skin cells in domestic rabbits, a histological study using a special dye

1. Introduction

The skin is the largest organ in the body, and plays a vital role in protecting the body from harmful environmental factors, as well as regulating temperature and sensation. Skin accounts for approximately 15% of the total body weight and is characterized by its ability to regenerate and repair (Burgess, 2015). The skin consists of three main layers: the epidermis, the dermis, and the subcutaneous tissue, and each layer plays a specific role in maintaining the integrity and functions of the skin (Kolarsick *et al.*, 2011) ^[1].

With age, all skin functions undergo significant changes. The skin's ability to regenerate is diminished, and signs of aging such as wrinkles and loss of elasticity appear (Farage *et al.*, 2008) ^[2]. The aging process affects the outer layer of the skin, making it more susceptible to damage and dryness (Makrantonaki & Zouboulis, 2007) ^[3].

Skin changes resulting from aging are not only the result of internal influences, but also chronic exposure to ultraviolet rays from the sun. Exposure to ultraviolet radiation accelerates the appearance of signs of aging, such as pigmentation and dark spots, which is known as photoaging (Krutmann, 2000) ^[4]. Oxidative stress from exposure to UV radiation and free radicals is thought to play a key role in this process, causing damage to DNA, proteins and lipids in skin cells (Fisher *et al.*, 2002) ^[5].

Understanding the biological changes that occur in the skin with aging is essential for developing effective strategies to maintain healthy skin. Recent research highlights the importance of prevention through the use of sun protection and daily skin care (Rigel, 2010) ^[6]. Furthermore, aging is associated with slower wound healing, which increases the importance of careful care for aging skin (Wong & Pomerantz, 2008) ^[7].

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Recent studies confirm that skin changes associated with aging are caused by a combination of genetic and environmental factors. Therefore, strategies focusing on the prevention of UV damage and oxidative stress, in addition to proper skin care, can be effective in reducing the effects of aging on the skin (Vierkötter & Krutmann, 2012)^[8].

Verhoff's collagen stain is a staining technique commonly used in histology to detect elastic fibers and collagen in tissues. This stain is an essential tool for identifying and evaluating ultrastructural changes in collagen-containing tissues, making it useful in the diagnosis of a variety of diseases and pathological conditions (Verhoeff, 1908)^[9].

Verhoff's collagen stain works by using a mixture of iron hematoxylin, ferrichloride, and thiourin. These compounds react with elastic fibers to produce a characteristic black color, while collagen is stained red to pink using another dye such as Van Gieson (Gordon & Sweet, 1936)^[10]. This technique allows a clear distinction between elastic and collagen fibers in tissues, which helps in the evaluation of structural and pathological changes.

Verhoff's collagen stain is particularly used in the diagnosis of diseases affecting connective tissue, such as atherosclerosis, cirrhosis, and fibrotic lung disease. For example, they can be used to determine the extent of damage to elastic fibers in arterial walls in cases of atherosclerosis, helping doctors assess the progress of the disease (Lie, 1959)^[11].

Furthermore, this dye is used in research studies to understand the changes that occur in connective tissue with age or as a result of exposure to certain environmental factors. Understanding these changes can help in the development of new treatments for diseases affecting connective tissues (Gamble & St. Mary, 1952)^[12].

The study aims to provide a comprehensive understanding of the histological and functional changes that occur in the skin of domestic rabbits with age and the extent to which aging significantly affects the structure and functions of the skin, which in turn reinforces the need to develop new therapeutic strategies to maintain skin health and delay signs of aging. It also aims to open the door to more research for a deeper understanding of the biological mechanisms that control the aging process and its effects on living tissues.

2. Materials and Working Methods

2.1 Collecting rabbits

The animals being investigated, namely local rabbits, were acquired with a total of 40 rabbits at various stages of age, encompassing early, medium, and long-lived individuals. The rabbits were gathered between August 2023 and February 2024, and were separated into seven groups.

Young and adult rabbits were acquired through the rearing and breeding of rabbits within the animal facility affiliated with the College of Veterinary Medicine/Tikrit University. Mature rabbits were sourced from reputable rabbit breeders, ensuring their age and disease-free status, and subsequently housed in dedicated enclosures for a period of time. Their health was regularly evaluated to assure their safety, considering laboratory circumstances such as ventilation, temperature ranging from 28 to 25 degrees Celsius, and a lighting schedule of 12 hours of light followed by 12 hours of darkness. The cages were lined with sawdust on the floor, and it was changed twice a week to maintain safety. The rabbits underwent a thorough examination by a veterinarian to verify their physical condition and appropriateness for the ongoing research.

2.2 Laboratory work

After the rabbits were acclimated into their seven groups, they were transferred to the surgical laboratory of the animal house at the College of Veterinary Medicine at Tikrit University for the purpose of dissection. Initially, the weights of the rabbits were recorded using a scale designed for small animals. The rabbits were then anesthetized with chloroform in preparation for dissection.

Hair was removed from the neck area, then the outer skin and inner membrane were opened. Next, the skin samples were transferred to Petri dishes containing normal saline (0.75%) for washing. As shown in Figure1, the washing process was repeated four times, alternating the solution each time to maintain its concentration. After obtaining clean samples free of blood residue, they were placed directly into sealed plastic vials containing a fixative solution to avoid damage and decomposition of the samples. Bottles are labeled with member name, lot number, and sample number to ensure accurate documentation.



Fig 1: A photograph showing the method of dissecting a rabbit

2.3 Microscopic Examination and Measurements

The rabbit's skin histological sections were analyzed using a Miotic light microscope at various magnification levels. Subsequently, the necessary portions of the histological sections were measured using an ocular micrometer.

2.4 Statistical analysis

Statistical analysis was conducted using the ready-made statistical program SAS system to calculate the significant differences between groups of study animals, by extracting the arithmetic averages for each group to compare with each other, then using one-way analysis of variance (ANOVA), as well as knowing the standard error (SE).

3. Results and Discussion

3.1 Laboratory results

• 2 months belly (A), ver

The epidermis contained a multi-folded paired squamous epithelium and the dermis contained a dense connective tissue composed of colloidal fiber bundles surrounding numerous hair follicles each containing a cortex and a medulla (Figure 2).

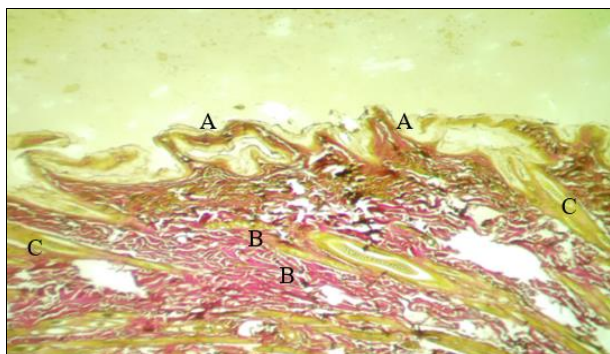


Fig 2: 2 months belly (A), ver. Epidermis of the skin and its multi-folded keratinized squamous epithelium (A) Colloidal fiber bundles in the dermis (B) Hair follicles composed of cortex and medulla (C) (Verhoff X10)

- **2 months belly (B), ver**

The subdermis layer contained dense connective tissue, surrounded by adipose tissue composed of broadly distributed fat cells, and around it was a subcutaneous layer containing skeletal muscle fibers surrounded by a dense connective tissue fascia (Figure 3).

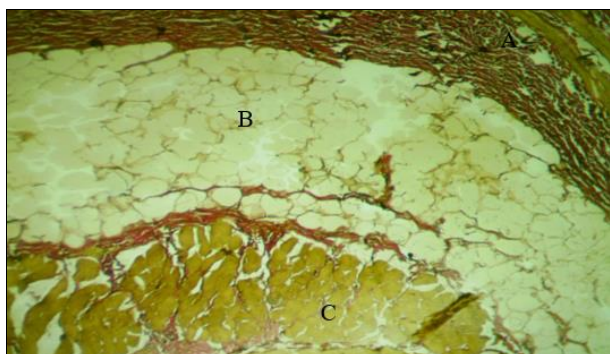


Fig 3: 2 months belly (B), ver. Subdermis layer with dense connective tissue (A) Adipose tissue (B) Subcutaneous skeletal muscle fibers (C) (Verhoff X40)

- **2 years Dorsal ver. – yellow color**

The epidermis contains paired squamous epithelium, the epidermis has outer folds, and the inner bandages with the dermis, forming human papillae - the dermis is composed of dense colloidal fiber bundles and has hair follicles extending toward the epidermis (Figure 4)



Fig 4: 2 years Dorsal ver. – yellow color skin epidermis composed of keratinized squamous cells (A) Epidermal folds and furrows (B) Epidermal papillae with dermis (C) Colloidal scout fiber bundles (D) Hair follicles (E) (Verhoff X40)

- **2 year Dorsal (A) ver**

The skin tissue contained degenerated epithelium, and the epidermis appeared with several pointed folds, with grooves emerging around those folds. The dermis contained hair

follicles in large numbers, oriented vertically towards the epidermis, surrounded by dense connective tissue (Figure 5).

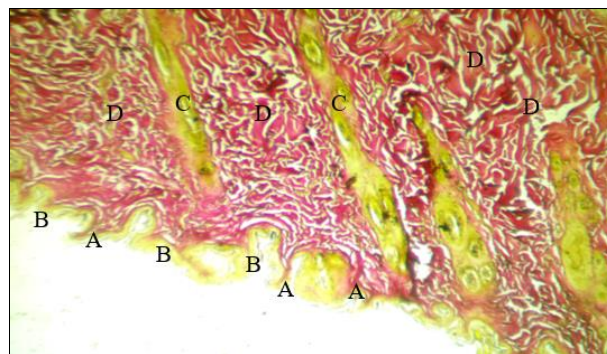


Fig 5: 2 years Dorsal (A) ver. Thin skin epidermis with many folds and ridges (A) Keratin layer (B) Hair shafts (C) Skin dermis with dense bundles of colloidal fibers (D) (Verhoff X10)

- **2 years Dorsal (B) ver**

The subdermis layer contains dense connective tissue adjacent to the adipose tissue, which contains degenerated fat cells, and under the skin, skeletal muscle fibers are found surrounded by fascia of colloidal fibers (Figure 6).

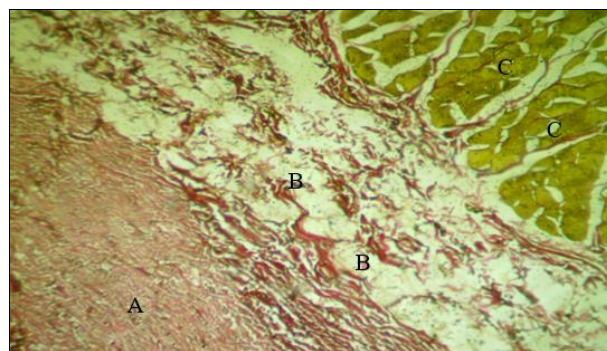


Fig 6: 3 years Dorsal (B) ver. Subdermis layer containing dense connective tissue (A) Adipose tissue containing degenerated fat cells (B) Subcutaneous layer containing skeletal muscle fibers in bundles (C) (Verhoff X10)

- **3 year Leg (A), ver**

The epidermis of the skin is surrounded by multi-rowed strands of keratin, and the epidermis contains degenerated, dark-colored epithelium. The dermis contains bundles of colloidal fibers. Some hair follicles are also found, consisting of a cortex with indistinguishable cells and a medulla that contains numerous follicles and vacuoles devoid of pigment (Figure 7)

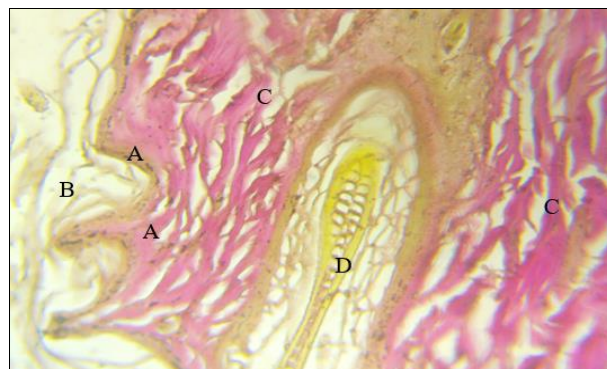


Fig 7: 3 year leg (A) Skin epidermis (A) Keratin rows (B) Colloidal fiber bundles (C) Cortex and medulla of hair follicles (D) (Verhoff X40)

- **3 year Leg (A), ver**

The dermis of the skin appeared wide, composed of bundles of colloidal fibers surrounding hair follicles spread throughout the entire dermis area, and the subdermis layer contained loose loose connective tissue fibers (Figure 8).

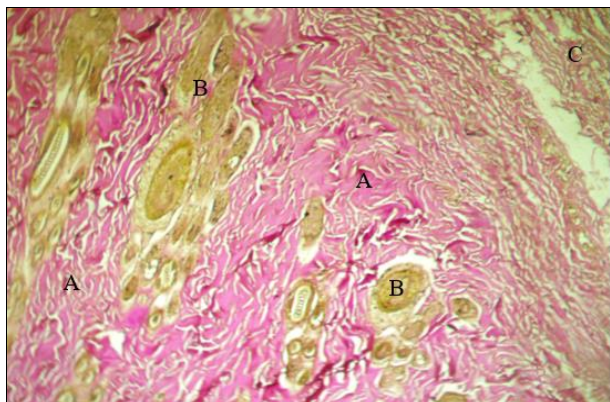


Fig 8: 3 years Leg (B) Dermis of skin and colloidal fiber bundles (A) Hair follicles (B) Subcutaneous layer (C) (Verhoeff X40)

- **8 months Dorsal (A) ver**

The dermis of the skin contained some hair follicles extending vertically towards the epidermis, surrounded by dense connective tissue with the presence of muscle tissue composed of skeletal muscle fibers interconnected from the subcutaneous layer (Figure 9).

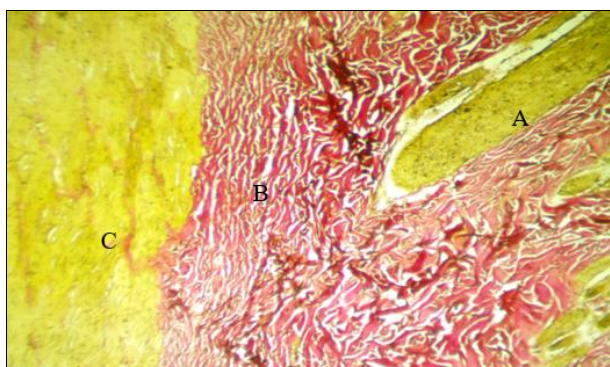


Fig 9: 8 dorsal month (A) Dermis tissue including hair follicles (A) Colloidal fiber bundles (B) Skeletal muscle tissue (C) (Verhoeff X40)

- **8 months Dorsal (B) ver**

The skin tissue contained a very narrow epidermis that appeared in the form of a thin strip, and hair follicles protruded from the dermis towards the epidermis and outside it through extensions, which are hair shafts, and around them from the dermis, bundles of colloidal fibers emerged (Figure 10)



Fig 10: 8 dorsal month (B) Tight epidermis (A) Hair follicles (B) Scouting colloidal fiber bundles in the dermis (C) (Verhoeff X40)

- **1 years Dorsal (A) ver**

The subdermis layer contained connective tissue composed of colloidal fibers separated from the subcutaneous layer by muscle fascia with dense connective tissue, and the subcutaneous layer contained rows of compact skeletal muscle fibers (Figure 11).

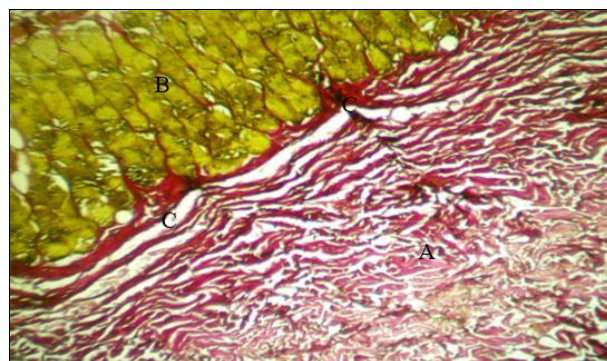


Fig 11: 1 dorsal year VER (A) Subdermal layer containing colloidal fiber bundles (A) Subcutaneous layer containing skeletal muscle fibers (B) Fascia muscularis (C) (Verhoeff X40)

- **1 years Dorsal (B) ver**

The epidermis of the skin is disintegrated and some of its parts have been shed, with the spread of many hair follicles in the dermis emerging from the epidermis in the hair shaft and connected to the sebaceous glands. The bundles of colloidal fibers in the dermis appeared moderately oriented and wavy (Figure 12).

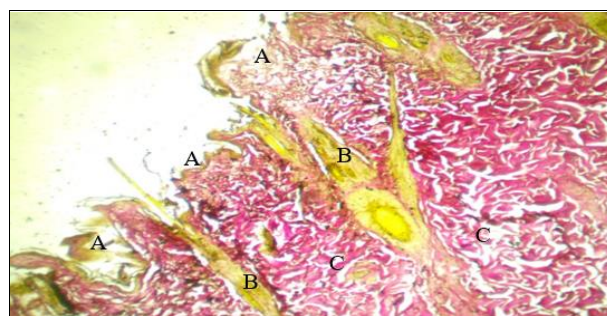


Fig 12: 1 year dorsal VER (B) Loose skin cuticle with some parts shed (A) Hair follicles extending deliberately hair from the dermis towards the cuticle and outside (B) Exploratory colloidal fiber bundles in the dermis (C) (Verhoeff X10)

3.2 Statistical analysis

- **M.C skin Belly: One-way ANOVA: Skin 2 Mon., 2 Month, 4 Month, 6 Month, 8 Month, 1 Years**
Analysis of Variance for

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	7	663.8	94.834	25.36	0.0008
Error	39	145.9	3.740		
Total	46	809.7			

Factor	N	Mean	StDev	95% CI
Skin 2 Mon.	5	4.600 d	1.140	(2.851, 6.349)
2 Month	6	11.000 b	1.414	(9.403, 12.597)
4 Month	6	9.670 b	3.27	(8.07, 11.26)
6 Month	6	17.000 a	1.414	(15.403, 18.597)
8 Month	6	7.000 c	1.211	(5.403, 8.597)
1 Years	6	4.667 d	1.414	(3.070, 6.264)
2 Years	6	10.000 b	2.83	(8.403, 11.597)
3 Years	6	11.000 b		(9.40, 12.60)
Pooled StDev =			1.93395	

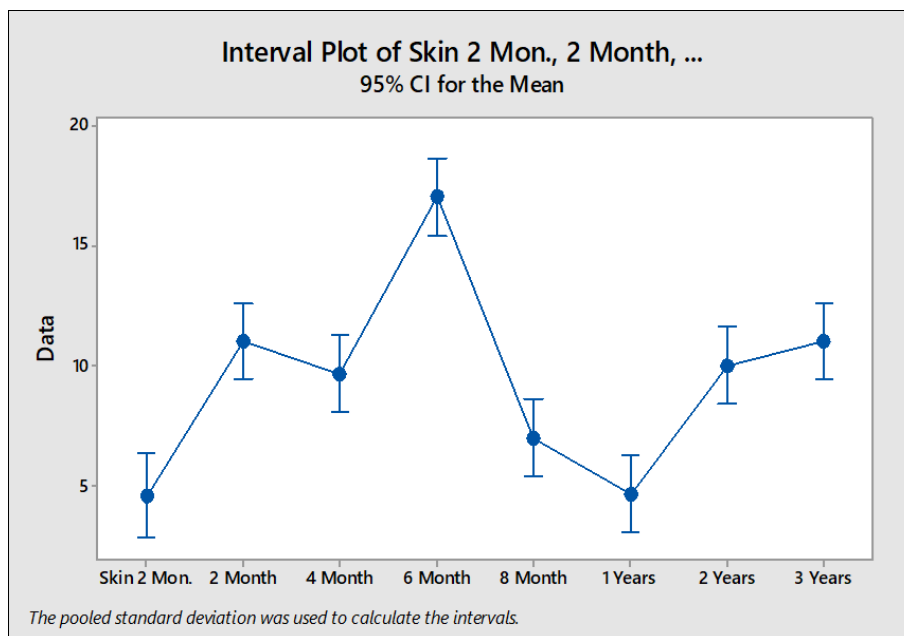


Fig 13: M.C skin Belly: One-way ANOVA: Skin 2 Mon., 2 Month, 4 Month, 6 Month, 8 Month, 1 Years

- **Skin Belly: One-way ANOVA: Skin 2 Mon., 2 Month, 4 Month, 6 Month, 8 Month, 1 Years, 2**
Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	7	13495604	1927943	106.40	0.00004
Error	42	760998	18119		
Total	49	14256602			

Factor	N	Mean	StDev	95% CI
Skin 2 Mon.	6	1200.0 de	70.7	(1089.1, 1310.9)
2 Month	7	750.7 g	6.73	(148.04, 353.39)
4 Month	6	1250.0 cd	7.07	(1139.10, 1360.90)
6 Month	6	1291.7 c	66.5	(1180.8, 1402.6)
8 Month	7	1868.0 b	32.6	(1765, 1971)
1 Years	6	1000.0 f	70.7	(889.1, 1110.9)
2 Years	6	1150.0 e	70.7	(1039.1, 1260.9)
3 Years	6	2000.0 a	70.7	(1889.1, 2110.9)
Pooled StDev =			134.607	

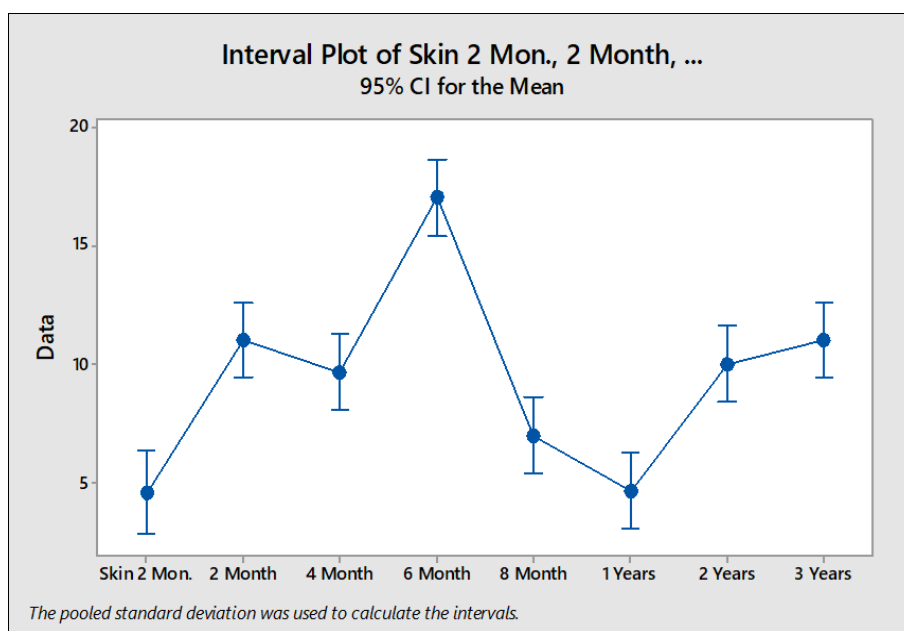


Fig 14: Skin Belly One-way ANOVA: Skin 2 Mon., 2 Month, 4 Month, 6 Month, 8 Month, 1 Years

4. Discussion and Results

4.1 Epidermal and Dermal Changes

- The study shows that aging leads to significant changes in the thickness and structure of the epidermis and dermis in rabbits. The thickness of these layers increases with age.
- In older rabbits, the epidermis contains degenerated epithelial cells, and there is an increase in the number of folds and grooves in the skin surface.

- The dermis shows a dense accumulation of collagen fibers, which becomes more pronounced with age. Hair follicles also become more numerous and oriented vertically towards the epidermis.

4.2 Subdermal Changes

- The subdermis in older rabbits shows a mix of dense connective tissue and adipose tissue, with signs of degeneration in the fat cells.

- Skeletal muscle fibers under the skin become more prominent and are surrounded by connective tissue, indicating structural changes in the subcutaneous layers.

4.3 Inflammatory Response and Vascular Changes

- Aging appears to trigger an increase in inflammatory cell activity within the skin, which could be linked to a decreased ability to repair and regenerate skin tissues.
- The study notes changes in blood vessel composition, including an increase in both size and number of vessels, which might affect skin circulation and, consequently, tissue nutrition and regeneration.

4.4 Collagen and Elastic Fibers

The use of Verhoeff's collagen stain highlighted changes in collagen and elastic fibers in the skin as rabbits age. The density and distribution of these fibers are altered, reflecting the reduced elasticity and increased rigidity of aging skin.

5. Conclusion

- **Aging Effects:** The study provides clear evidence that aging has a significant impact on the structural integrity and functionality of rabbit skin. These changes are similar to those observed in human skin, making rabbits a potentially useful model for studying skin aging.
- **Therapeutic Implications:** The findings suggest the need for developing therapeutic strategies aimed at maintaining skin health and delaying aging signs. This could include approaches to enhance collagen production, improve skin elasticity, and manage inflammation.
- **Further Research:** The study opens avenues for further research into the biological mechanisms behind skin aging, potentially leading to new treatments for age-related skin conditions.

Conflict of Interest

Not available

Financial Support

Not available

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