Original Contribution

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Forehead wrinkles: a histological and immunohistochemical evaluation

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Summary

Background Wrinkles are associated with cutaneous aging especially on sun-exposed skin. Despite they are considered a major topic in cosmetic dermatology, very few reports have studied the specific histological and immunohistochemical changes characteristic for wrinkles.

Aim The study aims to evaluate the histological and immunohistochemical changes of static forehead wrinkles in relation to surrounding photoaged skin.

Methods Biopsy specimens were obtained from the forehead wrinkles of 20 volunteers of Glogau's class III–IV wrinkles. Using histological and immunostaining methods coupled with computerized morphometric analysis, measurement of epidermal thickness and quantitative evaluation of total elastin and tropoelastin as well as collagen types I, III, and VII were performed for skin biopsies.

Results In the wrinkle site, there was statistically significant lower epidermal thickness ($P = 0.001$), elastin ($P < 0.001$), tropoelastin ($P < 0.001$), and collagen VII ($P < 0.001$) than the surrounding photoaged skin. Meanwhile, there was no significant difference between the wrinkle site and adjacent photoaged skin regarding collagen type I ($P = 0.07$) or III ($P = 0.07$).

Conclusion This study detected some histological and immunohistochemical differences in the wrinkle site when compared to adjacent photoaged skin. This may help in understanding the pathophysiology of facial wrinkling as well as its ideal way of management.

Keywords: collagen, elastin, extracellular matrix proteins, photoaging, skin aging, wrinkles

Introduction

Cutaneous aging, a complex biological phenomenon affecting different constituents of skin, is caused by two distinct processes: intrinsic and extrinsic.[1,2] One of the telltale signs of aging is increased wrinkling of the face, which are configurational changes in skin surface in the form of creases and furrows.[3] There are secondary factors causing these wrinkles of the face, including constant pull of gravity, frequent and constant positional pressure on the skin of the face (e.g., during sleep), and repeated facial movements caused by contractions of mimetic muscles of facial expression.[4]

Dermatologists can distinguish between two types of facial wrinkles: static and dynamic. Static wrinkles are always visible even when all facial muscles are resting

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as they developed in thin stretched skin as a result of premature or natural aging processes. Dynamic wrinkles, occurring in people of all ages even young children, appear temporarily when a muscle contracts causing the overlying skin to crease like an accordion. Accordingly, dynamic wrinkles are seen only when persons are animating their facial expression.[4]

The combined effects of chronological skin aging and photodamage can lead to degradation of collagen and accumulation of abnormal elastin in the superficial dermis, causing the skin to lose its thickness and elasticity and develop wrinkles.[5] Hence, skin that has lost its suppleness retains the lines formed by facial expressions such as smiling or frowning. Over a period of time, these "expression lines" deepen into wrinkles.[6]

Controversial data have been reported on the anatomy and histology of wrinkles with some authors reporting major skin changes specific for wrinkles,[7] whereas others report an almost identical histological aspect of the wrinkled skin compared to surrounding skin.[8] Moreover, there was no detailed information about the immunohistochemical changes specific for wrinkles.

This study aims to evaluate the histological and immunohistochemical changes of static forehead wrinkles in relation to the surrounding photoaged skin.

Material and methods

The study was conducted on 20 Caucasian Egyptian volunteers with forehead wrinkles, attending the Dermatology outpatient clinic of Al-Minya University Hospital for facial rejuvenation. This study was approved by the Committee for Postgraduate Studies and Research of Faculty of Medicine, Al-Minya University. Two dermatologists, independently, examined each volunteer to determine wrinkles type, according to Glogau's photoaging classification.[9] An informed consent was taken from each volunteer for taking biopsies.

Biopsy

From each volunteer, only one skin biopsy specimen, using 4-mm punch probe, had been taken from forehead wrinkles including photoaged skin on both sides of the wrinkles. The site of the wrinkle was marked because it was less apparent in the biopsy specimen due to release of the effect of hyperkinetic facial muscles. Each biopsy was fixed in 10% buffered formalin, embedded in paraffin, and sectioned perpendicular to the horizontal forehead lines into 5-μm-thick sections. These sections were stained with hematoxylin and eosin (H&E). Immunoperoxidase staining was performed using elastin and collagen I and III antibodies. Moreover, immunofluorescence staining was performed using tropoelastin and collagen VII antibodies. Light microscope [Accu-Scope # 3025 five headed (A3025-5); Olympus, Tokyo, Japan] with a built-in camera (digital camera E-330 SLR; Olympus) was used to examine and photograph the sections.

Histological measurements (histometry)

A computer-assisted program (analySIS® Five Olympus Soft Imaging Solutions GmbH, Johann-Krane-Weg 39, D-48149, Munster, Germany) was employed to measure both the epidermal thickness and the epidermal thickness under the wrinkle bottom in the same H&E-stained biopsy specimen, taken from the wrinkle site. The mean epidermal thickness was determined by measuring the distance between the outer most surface of the epidermis excluding stratum corneum and the dermo-epidermal junction at five points through the entire length.[10] Moreover, wrinkle width and depth were identified separately at five points with recording of their largest measures. To measure the width, a horizontal line was drawn between two points on both sides of the wrinkle and then the software calculated the measurement in μm. Meanwhile, for measuring the depth, a vertical line was drawn between the upper horizontal line and the bottom of the wrinkle, and consequently the software program automatically calculates the measurement.

Immunohistochemical staining

The immunoperoxidase technique was used to evaluate elastin (code no.: E4013, DBS, Sigma, St. Louis, MO, USA; at a dilution of 1:300), collagen type I (code no.: sc-59772, DBS, Santa Cruz Biotechnology, Santa Cruz, CA, USA; at a dilution of 1:400) and type III (code no.: ab6310, DBS; Abcam, Cambridge, MA, USA; at a dilution of 1:600).[11]

Indirect immunofluorescence (IF) staining was performed to evaluate tropoelastin (code no: GA317, Elastin Products, Owensville, MO, USA; at a dilution of 1:400) and type VII collagen (code no.: sc-33710, Santa Cruz Biotechnology, at a dilution of 1:600). For nuclear staining, tissues were incubated with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (code no: D8417, Sigma; at a dilution of 1:1000).[12] For each marker, a single-staining technique was used, and all specimens were stained at a single session.

The level of expression of elastin, tropoelastin, and collagen (types I, III and VII) was evaluated, by two blinded histopathologists. For quantitative evaluation of immune-stained tissues, a computer-assisted program was used. Using the histogram tool, a representative square was used to measure luminosity for fluorescent-stained sections, and color density for immunoperoxidase staining with obtaining a mean value, which was represented as percentage of expression.[10]

Statistical analysis

Data were statistically analyzed using the Software Package for Statistical Science (SPSS) (Version 16, Chicago, IL, USA). Statistical analysis included descriptive analysis as mean ± standard deviation (SD) for quantitative variables and frequency and percentages for qualitative variables. Independent sample *t*-test was performed for comparing the results between the wrinkle site and surrounding photoaged skin in the same biopsy specimen. Significance was expressed in terms of *P*-value, which was considered significant when it was ≤0.05.

Results

The study included 20 volunteers with horizontal static forehead wrinkles (11 females and 9 males). The age of these patients ranged from 45 to 60 years with a mean age ±SD of 52.05 ± 4.05 years. According to Glogau's photoaging classification, 17 persons showed wrinkles at rest (type III) and three persons had severe wrinkles (type IV).

Histological and histometrical results

Epidermal changes

The wrinkle width ranged from 30 to 89.1 μm with a mean of 62.9 ± 17.7 μm. Moreover, its depth ranged from 39.9 to 75.8 μm with a mean of 64.4 ± 12.2 μm. In the wrinkle site, the epidermal thickness ranged from 48.3 to 79.6 μm (mean, 64.2 ± 8.2 μm), which was statistically significantly lower when compared with adjacent photoaged skin (mean, 73.6 ± 8.6; $P = 0.001$), that ranged from 59.2 to 85.9 μm (Fig. 1).

Dermal changes

In photoaged skin, the most prominent changes occurred mainly in elastic and collagen fibers in all biopsies. Qualitative evaluation of dermal elastic fibers revealed haphazard distributions, in which the transverse plexus were seen clumped as densely stained elastotic material losing its fibrillar nature in the upper dermis and this material was separated from the epidermis by a thin unstained zone. In the wrinkle site, the elastotic material under the wrinkle bottom appeared less than those on each side of the wrinkle-forming real pads. This result was confirmed by quantitative evaluation of elastin, which revealed a statistically significant lower level at the bottom of the wrinkle when compared to nearby photoaged skin ($P < 0.001$). Moreover, tropoelastin showed a statistically significant lower level at the bottom of the wrinkle in relation to nearby photoaged skin ($P < 0.001$) (Table 1; Fig. 2).

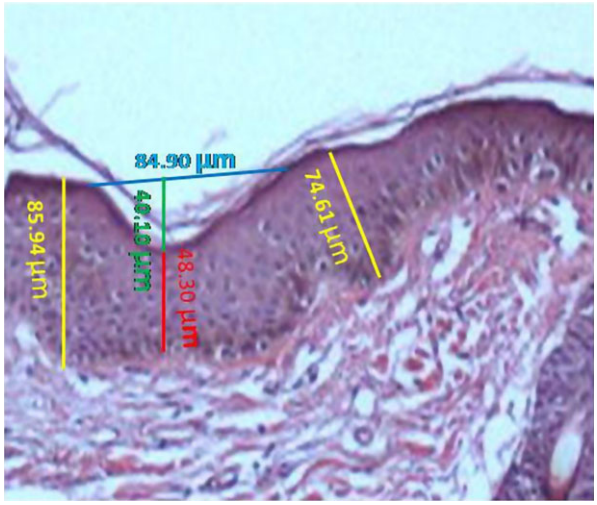

Figure 1 Histometry of skin biopsy specimen at wrinkle site showing significant decrease in the epidermal thickness at wrinkle bottom when compared to surrounding photoaged skin (H&E; 200).

As regards collagen fibers, they were distributed as randomly and loosely grouped fibers with clearly visible interfibrillary spaces throughout the whole dermis but tend to be more condensed just beneath the epidermis, in which densely stained band was seen with no fibrillar structure. With the advance of age, there is loss of collagen fibers so that only sparse amounts of collagen were seen in these areas. Quantitative evaluation of collagen I and III in the upper dermis demonstrated no statistically significant difference ($P = 0.07$) in their amounts in wrinkle site when compared to surrounding photoaged skin. Meanwhile, collagen VII showed statistically significant lower level ($P < 0.001$) at the bottom of the wrinkle in relation to nearby photoaged skin (Table 1; Fig. 3).

Table 1 Quantitative evaluation of elastin, tropoelastin, and collagen (types I, III, and VII) percent at wrinkle site and adjacent photoaged skin

	Elastin (%)		Tropoelastin (%)		Collagen I (%)		Collagen III (%)		Collagen VII (%)	
N = 20	Wrinkle site	Photoaged skin	Wrinkle site	Photoaged site	Wrinkle site	Photoaged skin	Wrinkle site	Photoaged skin	Wrinkle site	Photoaged skin
Range	39.7–55.2	44.6–77.5	12.2–16.6	15.9–27.8	54.8–59.6	56.8–59.9	50.2–58.4	54.9–58.7	10.8–13.2	14.6–19.9
Mean ± SD	49.6 ± 5.2	62.9 ± 8.1	14.02 ± 1.5	20.9 ± 2.9	57.5 ± 1.4	58.3 ± 1.02	55.8 ± 2.3	56.9 ± 1.26	12.03 ± 0.7	16.9 ± 1.6
P value	<0.001		<0.001		0.07		0.07		< 0.001	

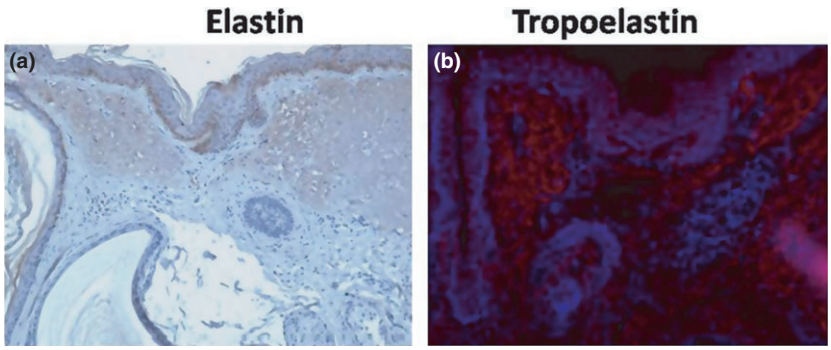

Figure 2 Dermal elastin and tropoelastin at wrinkle site. Immunoperoxidase staining of skin biopsy specimens for elastin (a) and immunofluorescence staining for tropoelastin (b) show significant lower level of elastin and tropoelastin at the bottom of wrinkle when compared to the surrounding photoaged skin. Nuclei stained in blue with DAPI in staining with tropoelastin (b) (original magnification 200).

Discussion

Wrinkles are folds of the skin, which are outward signs of intrinsic cutaneous aging appearing preferentially on sun-exposed areas (photoaging)[13] They are usually characterized by distinct microanatomical changes, and each type of wrinkle develops in specific skin region that is likely to respond differently to treatment.[14]

Estimation of the epidermal thickness in intrinsic cutaneous aging revealed slight overall thinning of the viable epidermis, which may be due to retraction of the rete pegs in most parts.[15,16] Initially in photoaged skin, the epidermis hyperproliferates in response to chronic ultraviolet rays (UVR) exposure with a resultant increase in epidermal thickness. In its later stages, UVR produces marked epidermal atrophy.[1]

At the wrinkle site, histometric evaluation demonstrated discretely thinned epidermis in its bottom compared with surrounding photoaged skin. This result agrees with that of Piérard *et al.*[17] This asymmetric variation may be explained by the difference of exposure to UVR. On the other hand, other investigators reported that there are no histological features distinguishing various wrinkles from surrounding skin.[18]

The normal dermis shows the elastic fibers, which are responsible for the resilient properties of the skin, constitute <2–4% of the extracellular matrix, and are dispersed throughout the dermis.[19] The histologic hallmark of photoaging is solar elastosis whereby the elastic fibers appear disorganized, thickened, and tangled. Elastosis most likely results from either UVR damage to the dermal fibroblasts causing them to secrete abnormal elastin or from low-grade digestion of extracellular matrix by proteolytic enzymes.[1,20]

At the wrinkle site, we observed a statistically significant lower level of elastin under the wrinkle bottom when compared to adjacent photoaged skin. This result agrees with some previous reports.[7,21] On the other hand, Kligman *et al.*[18] reported that there are no histological features that distinguish various wrinkles from surrounding skin. They concluded that the wrinkle is a configuration change, like the grooves worn into an

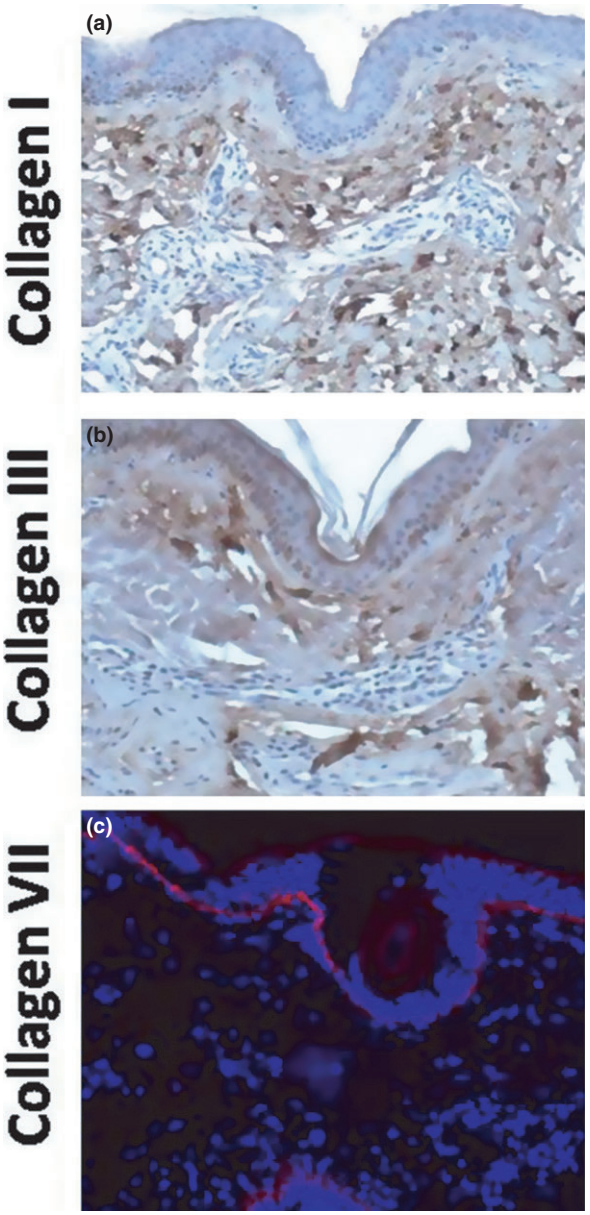

Figure 3 Dermal collagen types I, III, and VII at wrinkle site. Immunoperoxidase staining of skin biopsy specimens for collagen types I (a) and III (b) and immunofluorescence staining for collagen VII (c) show no significant change in collagen types I and III; however, collagen type VII was significantly lower at the bottom of wrinkle than the adjacent photoaged skin. Nuclei stained in blue with DAPI in staining with collagen VII (c) (original magnification 200).

old glove, without specific structural alterations at the histological level.

Elastic fibers are composed mainly of elastin, which is initially synthesized as tropoelastin.[22] The present study showed a statistically significant lower level of tropoelastin in the upper dermis at the wrinkle site when compared to the surrounding photoaged skin. On reviewing the literature, there was no previously published work concerning tropoelastin in facial wrinkles.

Collagen, a fibrous protein occupying about 80% of the dermis, is produced by the dermal fibroblasts and responsible for the tensile properties of the dermis allowing the skin to serve as a protective organ against external trauma. The most abundant type of collagen is type I collagen, which constitutes 80–85% of the collagen in skin, followed by type III collagen (10–15%).[23]

In aging process, gradual reduction of the amount of normal dermal collagen with fragmentation of its fibers occurs in photoaged skin,[1,24] which is compensated by compact elastotic material.[25] These findings were confirmed by *in vivo* assessment of aged skin using reflectance confocal microscopy showing loss of normal collagen fibers that are aggravated on photoaged skin areas,[2,26] with appearance of elastotic material in the upper and middle dermis.[27]

This decrease in amount of collagen may result from either increased degradation in sun-exposed skin[28] or reduced collagen synthesis.[29] However, El-Domyati *et al.*[1] noticed that there is a zone of densely stained collagen just beneath the epidermis, which could be still identified in the facial skin of elderly. On comparing the wrinkle site with the surrounding photoaged skin, there was no statistically significant difference between them regarding collagen type I and III. To the best of our knowledge, no previous work evaluated collagen I and III in wrinkle site compared to adjacent photoaged skin.

In the human skin, the stability of the dermo-epidermal junction is maintained in part by type VII collagen, the main component of anchoring fibrils, which is synthesized by both fibroblasts and keratinocytes.[30] In aging process, there is diminution of collagen VII, which may result from decrease in synthesis and/or an increased breakdown (by metalloproteinase).[31]

At the wrinkle bottom, collagen VII expression revealed a significant lower level when compared to the surrounding photoaged skin. This agrees with Contet-Audonneau *et al.*[21] and Craven *et al.*[31] This diminution in collagen VII at the bottom of the wrinkle compared to the flanks may contribute to the appearance of thin flattened dermo-epidermal junction,[21] weakening the bond between epidermis and dermis and accordingly formation of the wrinkle.[31,32] Meanwhile, the huge amount of adjacent elastotic material increases the magnitude of the wrinkles.[33]

It is apparent that the histological changes observed in photoaged skin, beside the repeated contractions of mimetic facial muscles, are among the main contributing factors for the development of facial wrinkles.[1,5,18,34] However, whether these changes observed, in the present study, at the site of wrinkles compared to adjacent photoaged skin are the primary cause, for genesis of frontal wrinkles, or a secondary event remains to be identified.

The authors are aware that one of the limitations of the present study is the relatively small number of volunteers. Nevertheless, it provided objective quantitative histological analysis of the skin changes in forehead wrinkles. However, further larger scale studies are needed to confirm and clarify such findings.

In conclusion, the present study revealed new details about the structure of static forehead wrinkles in the aging process. Histological evaluation of wrinkles bottom demonstrated discretely thinned epidermis as well as less elastotic changes, tropoelastin, and collagen VII when compared to the surrounding photoaged skin. Meanwhile, collagen I and III were not statistically significantly different in the wrinkle site compared to the adjacent photoaged skin. Identifying these changes may aid in better understanding of the pathophysiology of facial wrinkles and its ideal way of management.

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