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The use of Botulinum toxin-A injection for facial wrinkles: a histological and immunohistochemical evaluation

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Summary

Botulinum toxin (BTX)-A has been used for years in the reduction of facial wrinkles; however, histological and immunohistochemical changes after its use were not previously investigated. To evaluate histological and immunohistochemical changes after BTX-A injection for facial wrinkles, sixteen volunteers, with wrinkles on the upper third of the face, were subjected to single injection of BTX-A. Skin biopsy specimens were obtained from peri-orbital wrinkle site (crow's feet area) before and after 3 months of BTX-A injection. Using histological and immunohistochemical evaluation coupled with computerized morphometric analysis, measurement of epidermal thickness, wrinkle depth and width as well as quantitative evaluation of collagen types I and III and elastin was performed for skin biopsies. After BTX-A injections, there were significant increase in wrinkle width and granular layer thickness ($P < 0.001$), while the other histometrical measures as well as the immunohistochemical expression of collagen types I and III and elastin showed no significant difference ($P > 0.05$). However, collagen fibers showed better organization and orientation after BTX-A injection. The histological changes observed after BTX-A injection for facial wrinkles may help in better understanding of its mechanism of action.

Keywords: botulinum toxin-A, collagen, elastin, rejuvenation, skin aging, wrinkles

Introduction

Aging of the skin is likely caused by both intrinsic (biologic) and extrinsic (environmental) factors.[1] Clinically, skin features are greatly affected by aging as skin tends to become roughened, lax, and wrinkled.[2]

The main histological feature of photodamaged skin is the accumulation of elastotic material in the papillary and mid-dermis, a process known as solar elastosis. In addition, photoaged skin shows disorganized collagen network with gradual decrease in its content, which may be due to enhanced breakdown and/or decreased synthesis.[3,4] These changes contribute to the skin laxity and wrinkle formation.[5]

In addition to aging, there are secondary predisposing factors for facial wrinkles including pull of gravity, constant positional pressure on the skin (e.g., during sleep), and repeated facial movements caused by contractions of mimetic muscles of facial expression.[6]

Each type of wrinkle has characteristic microanatomical changes with variable response to treatment modalities.[7] Therefore, botulinum toxin (BTX)-A injection may be useful for wrinkles associated with mild degree of photodamage in patients with hyperkinetic muscles,

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while lasers and radiofrequency technology are more beneficial for more photodamage caused by loss of collagen.[8]

In spite of the expanding worldwide use of BTX-A injections for facial wrinkles with a relatively high total cost to patients, evidence-based publications discussing its effect at histological level are still lacking. Hence, this study aimed to investigate and objectively quantify the corresponding histological and immunohistochemical changes after BTX-A injection for facial wrinkles.

Methods

Subjects

The study was conducted on 16 volunteers, with wrinkles on the upper third of the face, attending the Dermatology Outpatient Clinic of Al-Minya University Hospital for the reduction of facial wrinkles. The study was approved by the Committee for Postgraduate Studies and Research of Faculty of Medicine, Al-Minya University. Two dermatologists, independently, examined each patient to determine the improvement and wrinkle types according to Glogau's photoaging classification.[9] An informed consent was obtained from each volunteer for treating with BTX-A injection, photographing, and taking biopsies.

Technique of injection

All volunteers were subjected to a single session of BTX-A for treating facial wrinkles on the upper third of the face (Botox® vial, 100 U of BTX-A, Allergan Pharmaceutical Company, Irvine, CA, USA). The techniques of injection and the dose were adjusted according to the site of wrinkles (horizontal forehead, glabellar frown, and crow's feet).[10] In case of horizontal forehead lines, 2 units (U) of BTX-A was injected at every point in three to four sites on either side of the midline with a total of 12–16 U. Meanwhile, the total dose of BTX-A in glabellar frown lines was 19–24 U, divided into 5–6 U for the procerus, 4–5 U for each of the medial corrugators, and 3–4 U for each of the orbicularis muscles. In case of crow's feet, 6–12 U (divided into 2 to 4 spaced sites with 2 or 3 U of BTX-A) of BTX-A was injected 1.5 cm lateral to the outer canthus (the lateral aspect of orbicularis oculi muscles).[10]

Biopsy

To standardize the results, punch biopsies (3 mm) were obtained from the same side, before and after 3 months of injection, from the peri-orbital site (crow's feet area), including the area surrounding the wrinkle. The post-treatment biopsy was taken from the same wrinkle site, at the nearest point to that of the pretreatment biopsy. The site of wrinkle was marked, as it was less apparent in the biopsy specimen due to release of the effect of hyperkinetic facial muscles. Each biopsy specimen was stained by hematoxylin and eosin (H&E), Masson's trichrome, and orcein. The immunoperoxidase technique was used for the evaluation of collagen types I and III, as well as total elastin. 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.

Histometric evaluation of wrinkles

A computer-assisted program (analySIS®Five Olympus Soft Imaging Solutions GmbH, Johann-Krane-Weg 39, D-48149, Munster, Germany) was employed in H&E-stained sections of biopsy specimens from the wrinkle site before and after 3 months of BTX-A injection. The mean epidermal thickness was determined by measuring the distance between the outermost surface of the epidermis excluding stratum corneum and the dermo-epidermal junction at 5 points through the entire length.[11] Moreover, epidermal thickness under the wrinkle and granular cell layer thickness were measured. Wrinkle width and depth were identified with reporting of their largest measurements.

Immunohistochemistry

The immunoperoxidase technique was used to evaluate collagen type I (code no.: sc-59772, DBS, Santa Cruz Biotechnology, Santa Cruz, CA, USA; at a dilution of 1:400), type III (code no.: ab6310, DBS, Abcam, Cambridge, MA, USA; at a dilution of 1:600), and elastin (code no.: E4013, DBS, Sigma, St. Louis, MO, USA; at a dilution of 1:300).[1,12] For each marker, a single staining technique was used.

The level of expression of collagen types I and III and elastin was evaluated, by two blinded histopathologists, using the computer-assisted program. Utilizing the histogram tool, a representative square 2.5×2.5 cm was used to measure the color density by obtaining the mean value, which represented the percentage of expression.[1,12]

Statistical analysis

Data were statistically analyzed using SPSS for Windows, version 16.0.1 (Chicago, IL, USA). Statistical

analysis included descriptive analysis as mean ± standard deviation (SD) for quantitative variables and frequency and percentages for qualitative variables. Paired-samples *t*-tests were performed for the results. Significance was expressed in terms of *P*-value, which was considered significant when it was ≤0.05.

Results

The study included 16 volunteers [8 women (50%) and 8 men (50%)], with wrinkles on the upper third of the face. The age of these patients ranged from 31 to 59 with a mean of 43.06 ± 8.44 years. Ten volunteers (62.5%) had type II wrinkle (wrinkles in motion), 5 volunteers (31.3%) showed type III wrinkle (wrinkles at rest), while only one volunteer (6.2%) had type IV wrinkles.

After 3 months of BTX-A injection, the efficacy of the injection was subjectively rated: (1) "very good" (10 volunteers, 62.50%), (2) "good" (5 volunteers, 31.25%), and (3) "poor" (1 volunteer, 6.25%). The total of "very good" and "good" scores was 93.75%. Peri-orbital wrinkles gave better results than horizontal forehead and glabellar wrinkles (Fig. 1).

Histological assessment

Epidermal changes

After BTX-A injection, there was a statistically significant increase in wrinkle width and granular layer

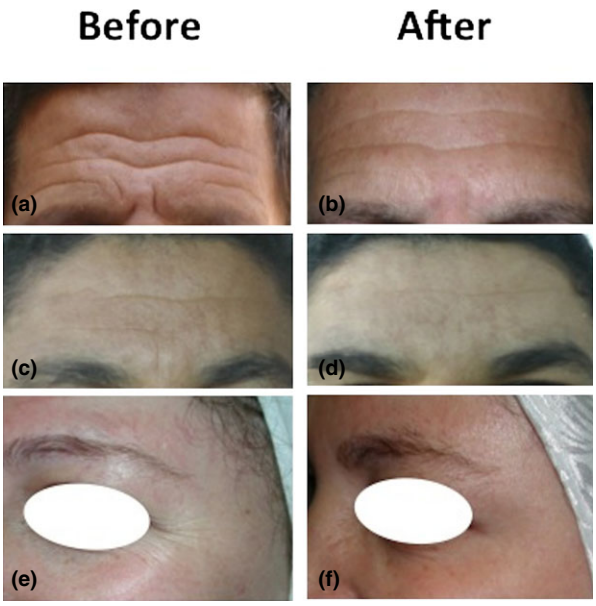

Figure 1 Clinical evaluation of volunteers in response to BTX-A. Representative photographs of forehead, glabellar (a–d), and peri-orbital areas (e, f), before (a, c, e) and 3 months after BTX-A injection (b, d, f).

Table 1 Histometric measurements before and after 3 months of botulinum toxin-A injection

	Epidermal thickness (μm)		Epidermal thickness under wrinkle (μm)		Granular cell layer thickness (μm)		Wrinkle depth (μm)		Wrinkle width (μm)	
	Before	After	Before	After	Before	After	Before	After	Before	After
Range	60.5–87.1	60.4–86.4	65.1–94.9	60.1–100.4	5.1–17.2	10.2–25.3	30–200	20.9–170.5	65–95.9	90.2–140.9
Mean ± SD	76.3 ± 8.6	77.1 ± 7.8	75.2 ± 8.9	77.5 ± 15.4	10.9 ± 3.3	16.5 ± 3.7	77.4 ± 52.2	70.9 ± 42.4	76.7 ± 10.2	112.3 ± 17.3
P-value	0.6		0.5		<0.001		0.08		<0.001	

thickness ($P < 0.001$), while the other histometrical measures including epidermal thickness ($P = 0.6$), epidermal thickness under the wrinkle ($P = 0.5$), and wrinkle depth ($P = 0.08$) showed no significant difference (Table 1; Fig. 2).

Dermal changes

As regards dermal changes after 3 months of BTX-A injection, the collagen bundles, which were disorganized with enhanced breakdown and reduced network formation in pretreated biopsies, became more organized and compact around the wrinkles with appearance of regular and smooth fibers. These findings were observed in 12 of 16 specimens (75%). Meanwhile, there was no significant increase in the density of collagen types I ($P = 0.2$) and III ($P = 0.4$). As regards elastin, elastotic material, which appeared as basophilic degenerated material in pretreated biopsy specimens with disappearance of the network of elastin microfibrils, did not significantly decrease after BTX-A injection ($P = 0.1$) (Table 2; Fig. 3).

Discussion

Controversy regarding the histology of wrinkles is still a matter of concern and under debate. Some authors reported major skin changes specific to wrinkles,[13] whereas others observed indistinguishable histological appearance of wrinkles from its surrounding skin.[7] In a recent study, our group detected significantly lower epidermal thickness, elastin, tropoelastin, and collagen type VII levels in forehead wrinkles compared to the surrounding photoaged skin.[14]

In the present study, we objectively quantified both the histological and the immunohistochemical changes of wrinkles in response to BTX-A injection. Data showed significant increase in wrinkle width and granular layer thickness, while the other histometrical measurements, including wrinkle depth, epidermal thickness, and epidermal thickness under the wrinkle, showed no significant difference. Our results agree with the theory supposing that BTX-A causes flaccid paraly-

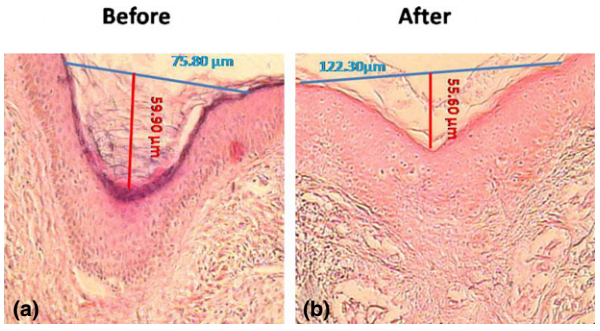

Figure 2 Histometry of skin biopsy specimens before and after 3 months of BTX-A, showing significant increase in wrinkle width, rather than decrease in wrinkle depth (b) when compared to baseline biopsies (a) (H&E; × 200).

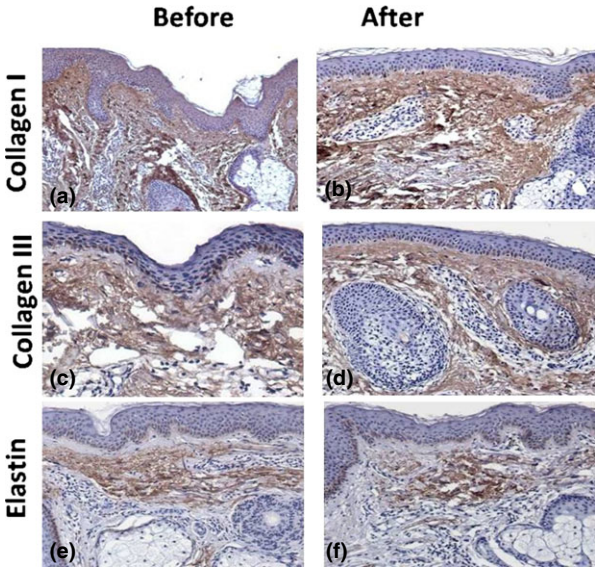

Figure 3 Dermal collagen and elastin before and after 3 months of BTX-A. Immunoperoxidase staining of skin biopsy specimens for collagen types I (a, b) and III (c, d) and elastin (e, f) showing no significant difference in collagen types I and III and elastin content between baseline (a, c, e) and post-treated biopsies (b, d, f), but with better organization and more compact collagen fibers after BTX-A injection (b, d) (original magnification; × 100).

Table 2 Quantitative measurements of collagen (types I and III) and elastin content at wrinkle site before and after botulinum toxin-A

	Collagen type I (%)		Collagen type III (%)		Elastin (%)	
N = 16	Before	After	Before	After	Before	After
Range	55.7–95.9	59.8–85.9	55.1–62.6	55.9–63.1	33.9–41.4	33.9–41.1
Mean ± SD	65.2 ± 9.2	66.4 ± 6.5	58.3 ± 2.2	58.6 ± 2.5	39.2 ± 1.8	38.9 ± 1.9
P-value	0.2		0.4		0.1	

sis in muscles by blocking the release of acetylcholine required for muscle contraction.[15] This muscle paralysis improves the appearance of wrinkles mainly by increased width rather than decreased depth.

The dermal matrix is composed mainly of collagen [type I (80–85%) and type III (10–15%)] in addition to glycosaminoglycans and elastic fibers.[16] After 3 months of BTX-A injection, the collagen bundles, which were disorganized with enhanced breakdown and reduced network formation, became more compact and organized around the wrinkle with appearance of regular and smooth fibers in most specimens (75%). Meanwhile, there was no significant change in collagen types I and III or elastin level.

The results of the present study showed that the clinical improvement observed after BTX-A injection was reflected at the histological level. Our findings gave further evidence that the improvement shown after injections may not be solely attributed to denervation of facial muscles, but also due to remodeling of the overlying tissues as suggested by Brandt and Fagien.[17] Moreover, this histological observation may explain the longer term improvement observed after repeated BTX-A injections.[17]

The authors are aware that one of the limitations of the present study is the 3-month time point of skin biopsy taking, with the possibility of decreased BTX-A action. BTX-A takes 3–7 days to achieve an effect and up to 14 days to reach its maximum effect that usually lasts for 4–7 months.[18] Thus, we selected that end point to give time to identify any histological change apart from the mere effect of muscle paralysis. However, further larger scale studies with multiple biopsies are needed to identify the sequence of changes occurring at different intervals after BTX-A injection.

In conclusion, the present study detected some histological changes after BTX-A injection for wrinkles such as increase in wrinkle width rather than decrease in its depth and increase in granular cell layer thickness with better organization and orientation of collagen fibers. These changes may help in better understanding of the different mechanisms of action of BTX-A injection for facial wrinkles.

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