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The Effect of Botulinum Toxin A on Fat Graft Survival

Rong Min Baek · Seong Oh Park · Eui Cheol Jeong · Hyun Sik Oh · Suk Wha Kim · Kyung Won Minn · Sam Yong Lee

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Abstract
Background Autologous fat grafting is a common procedure used in plastic surgery to correct soft tissue deficiency or depression deformity. However, absorption of grafted fat in the recipient area is unpredictable, and various methods for improving fat survival have been developed clinically. This study analyzed the changes and viability of injected fat in relation to the effects of botulinum neurotoxin type A (BoNTA).
Methods Fat tissue was harvested from the pre-urinary bladder cavity of four Sprague-Dawley rats and processed using the Coleman technique. The experiment was performed on the backs of eight BALB/c-nu mice. The injection of free fat grafts was performed on the bilateral side of the back of each mouse. The one side (experimental) was treated with 0.5 ml of a free fat injection combined with 0.5 IU of BoNTA in 0.1 ml of saline. The other side (control) was treated with 0.5 ml of free fat injection combined with 0.1 ml of saline. The mice were killed after 9 weeks, and the injected fat grafts were explanted, after which the weight and volume were measured. Histologic study was performed with hematoxylin and eosin staining. Statistical analysis of the weight and volume from both sides, the histologic parameters, and cellular integrity was performed.
Conclusion A difference in the weight, volume, and histologic parameters of the injected fat grafts was observed. The BoNTA-treated side exhibited a significantly higher survival rate than the control side. The histologic examination of the fat grafts also demonstrated that the grade scale of cellular integrity was higher for the BoNTA-treated sides. Botulinum toxin A significantly reduces the level of fat graft resorption. Therefore, an injected fat graft can be used in conjunction with botulinum toxin A and offers better volumetric improvement.
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Keywords Autologous fat grafting · BoNTA · Botulinum neurotoxin type A · Fat survival · Free fat graft

R. M. Baek · S. O. Park · E. C. Jeong · S. W. Kim · K. W. Minn
Department of Plastic and Reconstructive Surgery, Seoul National University College of Medicine, Seoul, Korea

E. C. Jeong (✉)
Department of Plastic Surgery, SMG-SNU Boramae Medical Center, 39 Boramae-gil, Dongjak-gu, Seoul 156-707, Korea
e-mail: ejeong.md@gmail.com

H. Sik Oh
Department of Pathology, Yangji General Hospital, Seoul, Korea

S. Y. Lee
Department of Plastic and Reconstructive Surgery, Chonnam National University Medical School, Gwangju, Korea

Autologous fat grafting was first attempted by Neuber in the late 1800s [1]. Since then, it has been used to correct depression sites due to aging or trauma. In the 1980s, Illouz [2] introduced liposuction. Since then, Coleman [3] has introduced methods for extracting autologous fat and procedures for a structural graft. Accordingly, autologous fat grafting using a fat injection currently is accepted as a standard method in the field of surgery [4].

Autologous fat is advantageous in that it is safe for the human body compared with alloplastic implants. A

sufficient amount of this ideal filler also can be obtained in an in vivo setting. However, it has the disadvantage that its effects cannot be accurately predicted due to a higher difference in absorption rates. Accordingly, in a clinical setting, overcorrections or retouch grafting are commonly performed to resolve this disadvantage [5, 6].

According to a review of the literature on various types of tissue transfer, graft tissue survives through revascularization. This survival is essential to maintaining the stable fixation of the graft at the recipient site in the early stage [7]. However, in facial fat grafting using a fat injection, the effect of initial fixation cannot be obtained because the fat is injected with a syringe into the facial region, where delicate movement of the face as in facial expression is persistently present.

The widespread use of botulinum neurotoxin type A (BoNTA) for cosmetic procedures have been effective for improving facial wrinkles by induction of muscular paralysis. Depletion of soft tissue volume and increased wrinkles are typical phenomena in the early stage of facial aging, and treatment has commonly been performed concomitantly with fat grafting [8]. This combination satisfies the patients' needs and the surgeon's aim.

Some surgeons, including the authors, have further favored the concomitant use of two treatment regimens under the speculation that the survival of the fat graft can be improved through its immobilization due to the effects of BoNTA in decreasing abnormal muscle contraction in the face. To date, however, most reports about the effects of this concomitant regimen have been made based on subjective descriptions of clinical experience by surgeons or clinicians. Due to a lack of objective reports based on experiments, we attempted to assess experimentally the effectiveness of the aforementioned concomitant regimen.

Materials and Methods

The study protocol was approved by the Laboratory Animal Use Committee of our institution. Four Sprague-Dawley rats (OrientBio, Seongnam, Korea) age 9 weeks and eight nude mice (BALB/c-nu mouse; OrientBio) age 6 weeks were used. The athymic nude mouse model was chosen because of its immunologic resistance to xenografts, and the rats were used for a fat source that was more easily accessible than human-oriented fat from a procedural standpoint. The animals were kept under standard laboratory conditions.

Experimental Animals and the Extraction and Purification of Fat Tissue

The adipose tissue of four Sprague-Dawley rats (300–350 g) age 9 weeks was extracted from the retroperitoneal fat layer and then chopped for fat grafting. The extracted fat tissue was centrifuged at 3,000 rpm for 3 min using the centrifugation technique of Coleman [3]. After the centrifugation, the mixture in a test tube contained lipid and cell debris in the superior part, an adipose cell layer with an abundance of adipose cells in the middle layer, and blood and fluid components in the lower layer. After the removal of the upper and lower parts, the fat tissue was harvested from the residual adipose layer.

The rat fat tissue still was fibrous and could not be further injected via a cannula. The fat tissue sample then was mixed with 2 ml of saline. With the addition of type 1 collagenase (1 ml), a homogeneous mixture was obtained. This was followed by a 2 h tissue degradation at 38.6°C. The degraded adipose tissue was centrifuged at 3,000 rpm for 3 min. With the lipid layer in the superior part excluded, the adipose layer in the middle part was harvested and placed in a 1-ml Luer-Lok syringe (Becton Dickinson, Franklin Lake, NJ, USA).

Fat Grafting and Injection of BoNTA

With the animal under isoflurane anesthesia, the fat was injected into the backs of eight nude mice age 6 weeks. The back area was divided into the left and right sides, and the skin was tapped by a 16-gauge needle, with the superior layer of back muscles exposed accordingly. At the site where the skin was tapped, a cannula was inserted, and the fat grafting was performed for the superior layer of the back muscles accordingly.

On the right side of the back region, the injection was done with a mixture of purified fat (0.5 ml), saline (0.1 ml), and BoNTA (0.5 IU) (BOTOX, Allergen Inc., Irvine, CA, USA). On the left side of the back region, the injection was done with a mixture of purified fat (0.5 ml) and saline (0.1 ml). Additionally, an injection of the same BoNTA dose (0.5 IU) was done in the superior region to the subcutaneous layer in the left gastrocnemius muscle. It was examined 3 days later to determine whether the effect of inducing muscle paralysis had occurred (Fig. 1).

Collection of Data on the Grafted Fat Tissue and Histopathologic Examinations

The presence of the fat graft 9 weeks after the grafting was grossly confirmed on the left and right sides of the mice backs, and then it was dissected (Fig. 2). The weight (g) of the fat graft was measured using an electronic scale (Ohaus, Pine Brook, NJ, USA). Graft volume (ml) was measured using the liquid overflow method based on the Archimedes principle of buoyancy. Thereafter, the extracted fat tissue was fixed in a 10% formalin solution. This was followed by preparation of paraffin-embedded tissue

Fig. 1 Fat transfer. The fat tissue is injected above the muscle on the left side of the back, and the 0.5 IU of botulinum neurotoxin type A (BoNTA)-mixed fat is on the right side of the back

sections, which then were stained with a hematoxylin–eosin dye. Using light microscopy, the number of adipose cells with a disrupted cell membrane and atypical cells with an elongated, columnar shape was counted and compared with the total number of normal adipose cells for a comparison of cellular integrity (Figs. 3, 4). The degree of cellular integrity was graded according to the proportion of adipose cells whose morphology was maintained near to a normal level as follows: 1 + (<5%), 2 + (5–25%), 3 + (25–50%), 4 + (50–75%), and 5 + (>75%).

Statistical Analysis

In the current experiment, the weight, volume, and degree of cellular integrity were measured and then expressed as mean ± standard deviation. Quantitative and qualitative analyses were performed based on a comparison of the weight, volume, and degree of cellular integrity. Statistical analysis using GraphPad PRISM 5.02 (GraphPad Software, La Jolla, CA, USA) was performed with paired *t* tests and Wilcoxon's signed-rank test. A *p* value less than 0.05 was considered statistically significant.

Results

Findings confirmed 3 days after the experiment that phalangeal abduction was incomplete due to paralysis of the gastrocnemius muscle in the left lower extremities where BoNTA was injected. This indirectly confirmed the expression of BoNTA, which was injected at the same dose concomitantly with fat grafting in the right back. A comparison of the weights between the control and the experimental sides showed a significant difference in the mean weights between the two sides (0.18 ± 0.086 vs. 0.35 ± 0.160; $P = 0.0391$) (Table 1). A comparison of the mean volumes of the two sides also showed a significant difference (0.22 ± 0.10 vs. 0.37 ± 0.14; $P = 0.0413$) (Table 2). When the degrees of cellular integrity were compared on the basis of histopathologic findings, the results showed an average of 2.9 ± 0.83 on the control side and 4.1 ± 0.99 on the experimental side. These results indicate that the experimental side had an excellent profile for mean degree of cellular integrity that reached statistical significance ($P = 0.0369$, Table 3).

Discussion

Correction of depression deformities due to aging or trauma has long been a major area of interest among plastic surgeons, and many methods for this correction have been introduced. One of these methods, autologous fat grafting, has been performed for a long time [1]. Autologous fat grafting with its related injection methods using a syringe-type cannula is considered a relatively simple procedure. However, it has the disadvantage that the survival of adipose cells cannot be predicted due to variability in the absorption rate depending on the grafted autologous fat tissue and the environment of recipient sites after the injections.

According to several previous studies, 20–90% of grafted fat tissue is absorbed. Due to this high rate of fat

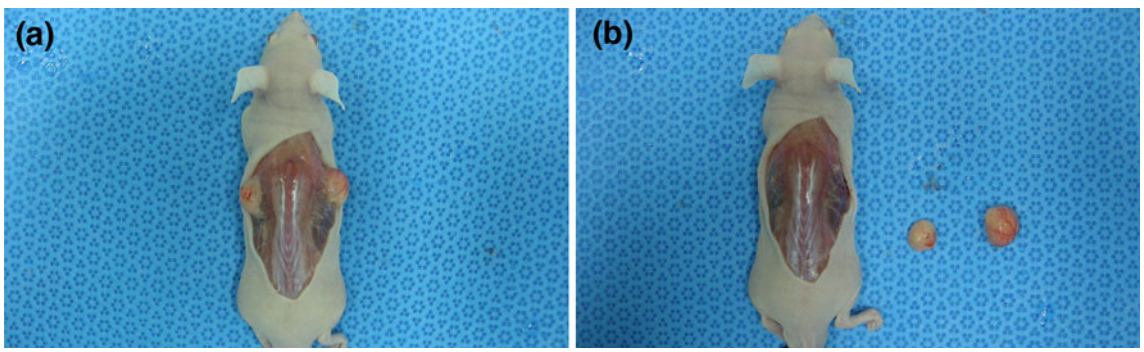

Fig. 2 Necropsy picture. **a** At 9 weeks postoperatively, the grafted fat specimen is removed. **b** The specimen from the right side is grossly larger than that from the left side

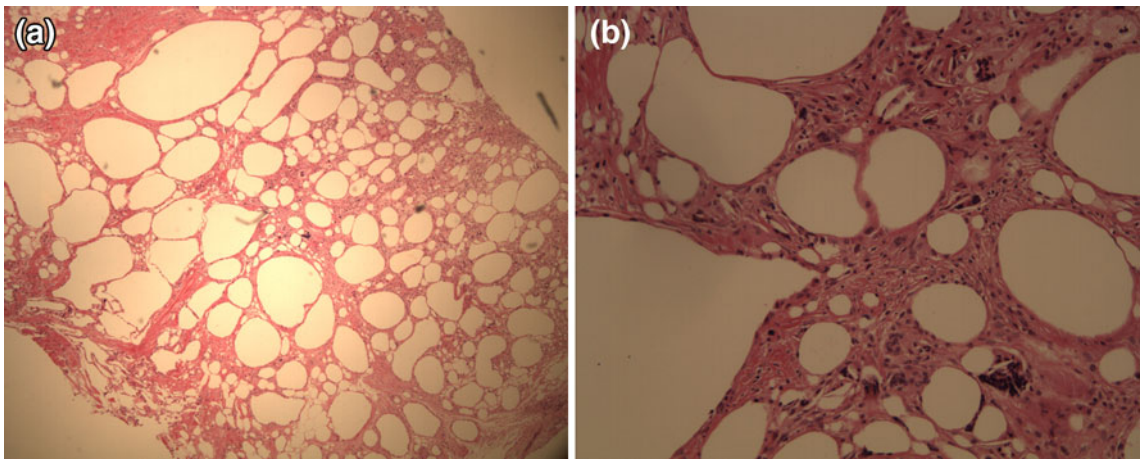

Fig. 3 Histologic appearance of the fat grafts from the control side without botulinum neurotoxin type A (BoNTA) (hematoxylin eosin stain). **a** It is assessed as cellular integrity grade 3+ at ×40 magnification. **b** There are diffuse histiocytic and neutrophilic infiltrates. Large vacuolar degeneration is shown to be extensive at ×200 magnification

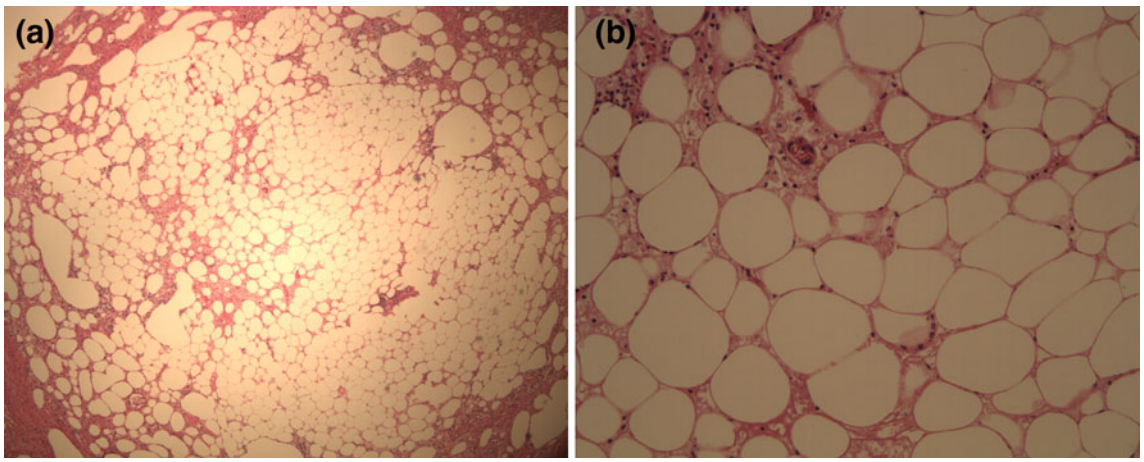

Fig. 4 Histologic appearance of the fat graft in the botulinum neurotoxin type A (BoNTA)-treated side (hematoxylin eosin stain) **a** It is assessed as cellular integrity grade 5+ at ×40 magnification. **b** The adipocytes with round vacuoles are well preserved, as seen at ×200 magnification

Table 1 Comparisons of weights (g) between the two sides

Mouse	BoNTA-treated side	Control side
1	0.32	0.16
2	0.25	0.13
3	0.46	0.14
4	0.10	0.06
5	0.16	0.31
6	0.50	0.21
7	0.47	0.16
8	0.50	0.30
Mean	0.35	0.18
SD	0.160	0.086

BoNTA Botulinum neurotoxin type A, *SD* standard deviation

$P = 0.0391$

absorption, overcorrection or retouch grafting is commonly performed to resolve the aforementioned disadvantages [5, 6]. This also is accompanied by the troublesome process collecting adipose cells for retouch grafting.

With regard to a theoretical basis for the survival of fat grafts, two opinions are widely accepted: (1) a host cell replacement theory maintaining that new adipocytes are formed in the host when the grafted fat tissue does not survive and that it is replaced by histiocytes, and (2) a cell survival theory maintaining that some grafted adipose cells are destroyed through a host response when histiocytes in the host play a role in scavenging the degraded lipid, and the residual adipose cells are left [9]. Recent studies have advocated both opinions based on the interaction between the fat graft and host tissue. That is, in both the fat graft and the recipient site, the host tissue, contain mature adipose

Table 2 Comparisons of volumes (ml) between the two sides

Mouse	BoNTA-treated side	Control side
1	0.40	0.20
2	0.40	0.20
3	0.40	0.20
4	0.10	0.05
5	0.20	0.30
6	0.45	0.20
7	0.50	0.20
8	0.50	0.40
Mean	0.37	0.22
SD	0.14	0.10

BoNTA Botulinum neurotoxin type A, *SD* standard deviation

$P = 0.0413$

Table 3 Comparisons of histologic cellular integrity grades (scales) between the two sides

Mouse	BoNTA-treated side	Control side
1	5	3
2	2	2
3	4	2
4	4	4
5	5	3
6	4	4
7	4	2
8	5	3
Mean	4.1	2.9
SD	0.99	0.83

BoNTA Botulinum neurotoxin type A, *SD* standard deviation

$P = 0.0369$; the cellular integrity parameter is graded on a semi-quantitative scale of 0 to 5 as follows: 1 (normal shape fat cells are <5%), 2 (5–25%), 3 (25–50%), 4 (50–75%), 5 (>75%)

cells and preadipocytes. Eventually, it can be presumed, the surviving fat graft is a mixture of mature adipose cells that survived after the transplantation and adipose cells that were differentiated from preadipocytes of host tissue.

Depending on the interaction between the recipient site and the fat graft, some grafted adipose cells are removed. It also is reported, however, that grafted cells stimulate the environment of the recipient site to induce the development of new adipose cells through differentiation and dedifferentiation [10].

In the current experiment, the adipose tissue was left for 9 weeks. Based on the aforementioned theories, this phenomenon can be interpreted to mean that the grafted adipose cells survived and then remained or that the adipose tissue was differentiated from adipose cells in the fat graft or host preadipocytes. But there is no evidence demonstrating which of the two responses was predominant.

In a study investigating the survival of fat grafts, Peer [11] maintained that all the cellular responses mentioned in both theories were observed. Furthermore, according to this author, the initial revascularization played a key role in the survival of adipose cells on day 4 after the transplantation. This author also noted that the surviving adipose cells were surrounded by fibroblasts and lymphocytes.

The importance of this revascularization also was underscored by Smahel [12]. According to this author, the degree of induced revascularization from the adjacent region was more important than which of the two cellular responses based on the two theories predominated for improved survival of fat graft. This might eventually have an effect on the long-term survival of fat grafts.

Before blood circulation via revascularization, nutrients are supplied to the graft based by diffusion from the plasmic circulation. To ensure that this action occurs, recipient sites must have abundant blood circulation and a lack of infections. Besides, to make sure that the fat graft has good contact with the recipient site as widely as possible, stable fixation of the fat graft is an essential factor for skin and other tissue transplantation [3, 7]. However, under the subcutaneous layer where the fat is transplanted to correct the soft tissue deformity, there are muscular tissues. With the actions of these muscular tissues, the initial stable fixation of the fat graft can be impaired. Due to the contraction of adjacent muscles, the fat graft is subject to physical compression.

With regard to body areas subject to voluntary control, patients should be educated about appropriate management. In the extremities, where no stiffness can be caused with the use of a splint or an external pin fixator, the survival of the grafts can be assisted with fixation during a certain length of time within a range [7]. However, when fat is grafted in facial regions, it must be remembered that although facial mimetic muscles distributed in the face are voluntary muscles, they are not commonly subject to arbitrary control. It also is impossible to apply external devices such as a splint to the face.

Botulinum toxin is a neurotoxin secreted from *Clostridium botulinum,* whose mechanisms are based on suppression of the fusion of acetylcholine vesicles to the membrane at the terminal of the neuron axons. If the target organ is a muscle, BoNTA acts on the neuromuscular junction, and this leads to blocking of nerve conduction. This inhibitory effect appears within several days and persists for a transient period ranging from 3 to 6 months. Using these effects from the induction of muscle paralysis, BoNTA has long been applied for the treatment of such diseases as muscle spasm and blepharospasm. The effects of BoNTA in selectively weakening the functions of facial expression muscles for therapeutic purposes and thereby improving facial wrinkles are widely recognized. As a

result, its indications have been extended to the areas of cosmetic procedures. Accordingly, it frequently has been used as a nonsurgical treatment regimen for facial rejuvenation [13].

To maximize the survival of injected fat grafts, many studies have examined the methods for the extraction and injection of fat. Furthermore, to the extent that the morbidity and the quantitative limitations of the donor site can be overcome, even tissue-engineering studies have been conducted using differentiated adipose cells and adipose-derived stem cells [14, 15]. But most of these studies carry a high cost and cannot be applied to a clinical setting or their procedure or manipulations are somewhat complicated.

As described in this report, autologous fat grafting for the correction of depression deformity based on the filling effects and the use of BoNTA for the improvement of facial wrinkles is very familiar to plastic surgeons [8]. Accordingly, a concomitant use of these two treatment methods is less invasive than a face-lift. Therefore, it is a common approach for patients in need of a prompt recovery.

Commonly, BoNTA injections are first performed and then followed by the fat grafting approximately 1 week later. But in some cases, the fat grafting and BoNTA injections are concomitantly performed. Within 2–7 days after BoNTA injections, the muscle paralysis is promptly induced. Accordingly, the clinical outcomes do not differ significantly between the concomitant use of these two treatment regimens and the single use of each one. The concomitant use of two treatment regimens is therefore universal for the convenience of patients.

In the face, small muscle tissues are densely arranged adjacent to the skin. In addition to conventional BoNTA injections, the use of dermal or intradermal injections of various BoNTA doses might effectively increase the skin tone and meticulously improve facial wrinkles [13]. This treatment regimen further powerfully induces paralysis in the muscle attachment on the superficial layer, with actions of diffusion to adjacent regions, leaving mobility to the deep-layer muscles. Thus, the mobility of facial expression muscles is preserved. This contributes to more natural facial appearances.

In this study, the concomitant use of BoNTA with the fat grafting reduced the functions of back muscles via the diffusion effects. Within the first 4 days, during which the fat graft underwent revascularization, it produced an effective immobilization, eventually leading to a stable contact. The graft was therefore offered a more stable environment by a decrease in neighboring muscle contractions and a higher residual graft volume via accelerating angiogenesis.

The review of the literature related to BoNTA identified some articles that introduced the lipolytic effect of BoNTA [16, 17]. But they are another story because they dealt with the anatomically organized subcutaneous fatty layer. The grafted fat was of heterotrophic origin from other anatomic areas. Angiogenesis is the most well-grounded concept in the surviving graft before everything else.

Other recent studies on BoNTA show that in addition to its effects on the neuromuscular junction, BoNTA also reduces the secretion of norepinephrine and thereby improves vasoconstriction. However, due to a lack of effects on the secretion of nitric oxide involved in vasodilation, BoNTA is effective in promoting vasodilation. Furthermore, experimental studies also have shown that a local use of BoNTA for the elevated flap raised the survival of a flap through the peripheral vasodilation based on the inhibition of the autonomic nervous system [18–20].

Kim et al. [21] found an abundance of markers associated with vasodilation and proliferation of vascular endothelial cells in a qualitative analysis using the polymerase chain reaction (PCR) in the flaps treated with BoNTA. They observed improvement in the hemodynamic status through the angiogenesis as well as the induction of vasodilation. These authors also speculated that the effects of BoNTA in improving the hemodynamic status might also contribute to the revascularization of fat grafts. However, no accurate mechanisms can be further discussed in the current status for two reasons. First, further studies are needed to examine the process of fat graft survival on a cellular level. Second, our results were obtained from the skin of mice whose structure is quite different from that of human skin. A simple comparison cannot explain all the findings. Further double-blind clinical studies are needed for the accurate assessment.

Summary

In an attempt to assess the effects of BoNTA injection used concomitantly with fat grafting via a fat injection, the current study compared the survival of fat grafts between a group for which a fat injection alone was performed and a group for which the fat injection was performed concomitantly with BoNTA injections. After a concomitant use of fat grafting with BoNTA injections, the gross weight and volume of the fat grafts and the histologic degree of cellular integrity were significantly higher than those of control subjects ($P < 0.05$). This provides the experimental basis for concluding that a concomitant use of fat grafting with BoNTA injections produces more effective treatment outcomes for the correction of facial deformities.

Conflict of interest No conflicts of interest exist and potential investigator conflicts of interest were not disclosed to study participants.

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