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EFFECT OF THE BOTULINUM TOXIN ON SURVIVAL RATE OF FAT TISSUE GRAFT (AN EXPERIMENTAL STUDY)

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ABSTRACT

INTRODUCTION: Enhancing the survival rate of fat grafts is still a challenging issue. Therefore, the introduction of additives such as Botulinum Toxin (BoNTA) to promote fat graft survival was taken into consideration and initially assessed in a rabbit model.

Aim of the study: Is to evaluate histologically and histomorphometrically the effect of BoNTA on the survival rate of fat graft.

MATERIALS AND METHODS: We used 15 rabbits in each group. Each rabbit had two incision lines made in its lips as recipient sites, and fat tissue was extracted from the donor site—the scapular adipose sacs—by cutting those incision lines. There were two sets of lips on each of the rabbits: one set was for the control group, and the other study group. The control group merely received a fat transplant with 0.5ml of saline, while the experimental group received a fat graft with 5 units of BoNTA. Five rabbits were scarified at the 3, 6, and 9-week postoperative testing intervals. The upper lip was dissected for a light microscopic analysis and histomorphometrical investigation.

RESULTS: Histological evaluation showed that injection with BoNTA improved survival rate and vascularization of fat graft. In comparison to the control group, the study group's percentage of the surface area of the fat graft rose statistically significantly at 3, 6 and 9 weeks (P <0.001), according to histomorphometrical analysis.

CONCLUSION: The current experimental investigation implies that pre-transplantation treatment with BoNTA can enhance the integrity and angiogenesis of fat graft.

KEYWORDS: Fat graft, Botulinum toxin, Rabbit model.

RUNNING TITLE: BoNTA effect on fat graft survival rate

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INTRODUCTION

When it comes to soft-tissue fillers, the fat tissue is the best option due to its biocompatibility, availability, non-allergic and non-toxic qualities, also its harmony with the natural texture of skin and soft tissues, and natural source where it is often abundant (1).

The engraftment of adipose tissue in the reconstructive surgeries can provide a viable alternative for filling any asymmetrical defects and promotes the spontaneous regeneration of soft tissue. In the report by Neuber (1893) had the first attempt of applying the adipose tissue engraftment (2).

Autologous fat transplantation has been widely applied for the soft tissue filling, cosmetic surgery for volume restoration, scar revision, and correcting craniofacial deformities, such as closing oro-antral communications and using a versatile pedicle graft for closing postoperative maxillary and mandibular defects (3).

The rapid decline in adipocyte count is considered the primary factor contributing to the minor survival rate of fat transplantation. Actually, this rapid decline occurs within 12 hrs (4). After the implantation of fat particles due to ischemia and inadequate nutrition which causes both necrosis and fibrosis. Accelerating early vascularization and promoting pre-adipocyte differentiation by including more materials into the graft is thus one of the primary strategies for raising the survival rate (5).

Nevertheless, due to wasting about 20–50% of the graft's volume after transplantation, surgeons have been hesitant of using the autologous fat as grafts (6).

Currently, autologous fat transplantation is a well-known medical technique. Numerous animal models were suggested in the literature to investigate this procedure. However, the major issue was that the area where the fat graft transplanted exhibited an inflammatory response with focal areas of necrosis (7).

Numerous factors, such as the donor area, the recipient area, the applied method for harvesting the targeted tissue, and the given care to the fat tissue prior to transplantation, all have an impact on the long-termed survival of the transplanted tissue. So in order to improve the survival rate, many scholars have attempted to carry out basic and clinical researches (8).

BoNTA is a highly neurotoxic protein made during reproduction by the bacterium Clostridium botulinum. In clinical settings, such as the treatment of headaches and the elimination of facial wrinkles and scars, boNTA injection is frequently used. Previous studies had shown that the use of botulinum toxin can increase the survival rate after fat transplantation, and they have produced promising results (9).

Therefore, the current report was designed in order to study the fat graft survival rate when injected with BoNTA in rabbit upper lip model. The hypothesis is that the BoNTA when combined with fat graft promote the efficacy of autologous fat transplantation and increase the survival rate of it.

MATERIALS AND METHODS

Study sample and setting

Our current study was carried out after having the approval of the research ethics committee, Faculty of Dentistry, Alexandria University.

In the study, fifteen male Egyptian Rabbits were contained for each group. The rabbits' mean age was one year and the average weight was (3.5 – 4) Kg. The animals were obtained from the animal house of Medical Research Institute, Alexandria University. The method needed to determine the proportional difference in the fat survival weight between control group and study group was the split half method; taking into consideration 95% confidence level and 80% power using Chi Square-testing (10, 11).

The animals were kept under normal ventilated laboratory conditions of temperature (22-25°C). During the course of the trial, the rabbits were housed in cages with a wired mesh bottom that were specifically made for allowing the suitable ventilation. The animals were fed and given water daily. Our study had the approaches that acomplished no or the least pain or stress to the subjected animals (12).

In control rabbits, the recipient site was on one side of the upper lip, whereas the other side was used for the other study group. Fifteen incisions were grafted in the lips with the fat tissue injected with saline and the other fifteen contra-lateral defects were grafted with fat tissue injected with BoNTA.

A coin toss was applied for determining which side of the animals to be used as study group and which was control group.

Materials

Purified Botulinum Toxin type A [**Siax, by medy tox (korea)]**, vial contain 100 units of clostidium botulinum neurotoxin type A.

Methods

All the operative procedures were performed under general anesthesia. The animals were anesthetized by an intramuscular injection of (0.15-0.20mg)/Kg ketamine plus (1-2mg)/Kg lidocaine (12).

The surgical area located below the scapula bone and all the rabbits' lips were shaved before any taken procedures. The rabbits 'skins were rinsed and scrubbed with 2% povidone iodine for avoiding any contamination. The surgical blades used were No. 10. Then, an incision was made along the dorsal middle line slightly below the scapula bone, which allows two fat fragments to be surgically separated (13).

The recipient site was prepared by using the surgical blade No. 10 to make a small posterior incision (2 cm) and marginally superior to the oral commissure. Conventionally, for the control, the recipient site was on one side of the upper lip. While, the recipient site for the study group was on the other side. The pre-tunneling level within the musculature of the superficial upper lip and its extension medially to the median upper lip cleft were both present in the top lip. Saline-injected adipose tissue was grafted into one side of each rabbit's upper lips, while the adipose tissue that had received five units of BoNTA was grafted into the other side of the upper lip. Each incision was sealed with a suture material. All the rabbits received the same course of antibiotics amoxicillin 1gm/Kg body weight every eight hours for five days (14, 15). **Figure (1)**

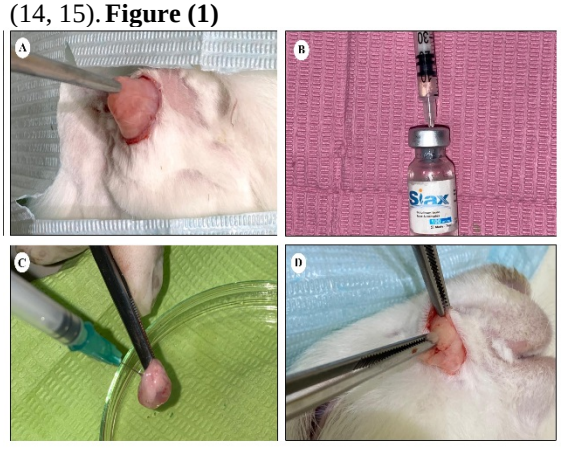

Figure (1): Fat graft harvested from scapular region then transplanted in the upper lip after injected by BoNTA

Animal euthanasia (16)

At the experimental time points of 3, 6, and 9 weeks postoperatively, five rabbits were overdosed to be euthanized with ketamine [15] The rabbits' lips were collected. For Histomorphometric analysis and light microscopic examination, the upper lip was removed and processed.

Histological examination (17)

Specimens were cleaned after being preserved in 10% neutral buffered formalin. The samples were cleaned, dehydrated by using ascending grades of alcohol, clarified in xylene, and then infused with and embedded in paraffin wax. Serial slices of 5 m thick were cut from the blocks of paraffin using a rotary microtome. Then, these paraffin blocks were stained with hematoxylin and eosin.

Histomorphometric analysis (18)

In our work, we decided to measure the proportion of surface area occupied by fat because it is considered the utmost indicative parameter for the evaluation of a fat tissue graft. The Image J 1.46r application was employed to calculate the morphometric evaluation of the percentage of surface area occupied by fat tissue. Each specimen had 2 longitudinal sections taken at various standard depths. An image was captured with the same magnification from each section (H&E, × 100, × 400). Each image's fat graft area was chosen using the free-hand selection tool, measured, divided by the rectangle's overall area, and multiplied by 100 to get the proportion of the rectangle that is made up entirely from the fat tissue.

The obtained results were presented as percentages (The proportion of area occupied only by fat in relation to the total surface area of the field). The means were calculated by repeating the same process for each of the two portions of the same specimen. The five specimens in each group underwent the same process five times.

Both the means and standard deviations were applied to describe the obtained data from the Histomorphometrical investigation in a statistical manner. In order to compare the values between the various groups, the analysis of variance (ANOVA) test was utilized. The significance threshold (p = 0.05) was. Statistics were significant for values under 0.05. The Statistical Package for Social Sciences (SPSS) version 20.0 was applied to conduct the statistical analysis.

Statistical analysis of the data

With the assistance of the IBM SPSS software package version 20.0, the computer was fed the information to analyze it. **(IBM Corp., Armonk, New York)** Also, number and percentage were used to describe qualitative data. The normality of the distribution was examined using **The Shapiro-Wilk test**. The range (minimum and maximum), mean, standard deviation, median, and interquartile range were used to characterize quantitative data (IQR). At the 5% level, the obtained data significance was evaluated. To compare between more than two groups using normally distributed quantitative variables, **the F-test (ANOVA)** is used, and the Post Hoc test **(Tukey)** was used for pairwise comparisons. To compare two eras, the **paired t-test** was employed with normally distributed quantitative data.

RESULTS

Histological results

Light micrograph (LM) examination of specimens at 3 weeks showed in both control and study groups, were densely populated by well-defined adipocytes and showing some blood vessels. **(Figure 2)**

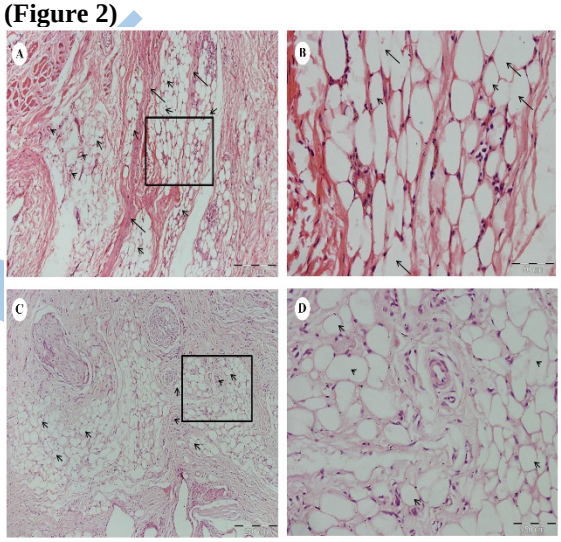

Figure (2): Light micrograph (LM) of the graft area of control group (A and B) and study group (C and D) at 3 weeks. **A:** shows the fat graft of control group with numerous adipocytes (short arrows). Fibrous tissue is seen between fragments of the graft (Long arrows). Some blood vessels are also seen (arrow head). **B:** (higher magnification of the previous micrograph inset) shows adipocytes with well-defined borders and eccentric nuclei (short arrows). Other areas show rupture of adjacent adipocytes (long arrows). **C:** shows the graft of study group which is densely populated by fat cells (arrows). Small blood vessels are seen in the connection tissue septa (arrow head). **D:** LM of higher magnification of the previous micrograph inset showing the cellular architecture of adipocytes (arrows). Rupture of few adjacent adipocytes is seen (arrow heads). (H&E, A and C × 100, B and D × 400)

Histological examination at 6 weeks showed more degeneration of fat cell in the control group, but in study group adipocytes showed preserved architecture with numerous blood vessels. **(Figure 3)**

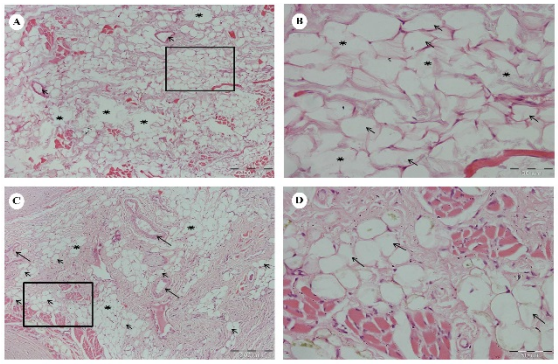

Figure (3): Light micrograph (LM) of the graft area of control group (A and B) and study group (C and D) at 6 weeks. **A:** shows areas of degeneration (stars) in the graft of control group. Some blood vessels can also be seen (arrows). **B:** (higher magnification of the previous micrograph inset) shows partial loss of the normal architecture of adipocytes. The cell borders are ill defined (arrows). Other areas show rupture of adjacent adipocytes (stars). **C:** shows the preserved architecture of adipocytes (short arrows) of study group with presence of few areas of partial degeneration of fat cells (stars). Numerous blood vessels is seen (long arrows) in the connective tissue septa. **D:** (higher magnification of the previous micrograph inset) shows the preserved shape of adipocytes. Some fat cells shows ill-defined border (arrows). (H&E, A and C × 100, B and D × 400)

In 9 weeks, the histological examinations revealed that the control pronounced loss of adipocytes normal structure and with no visible blood vessels. In contrast, the study group's specimens showed fat cells with preserved shape and blood vessels were obvious. **(Figure 4)**

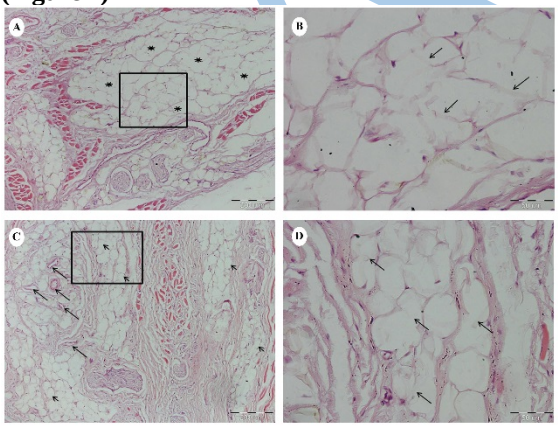

Figure (4): Light micrograph (LM) of the graft area of control group (A and B) and study group (C and D) at 9 weeks. **A:** shows the pronounced loss of the normal structure of the fat graft of control group. Most of adipocytes showed degeneration leaving large spaces (stars). **B:** (higher magnification of the previous micrograph inset) shows the marked disruption of adipocytes cell boundaries (arrows). **C:** shows the graft of study group is still populated by many adipocytes with preserved architecture (arrows short). Numerous blood vessels can be seen between adipocytes (long arrow). **D:** (higher magnification of the previous micrograph inset) shows few areas with partial disruption of adipocytes cell membrane (arrows). (H&E, A and C × 100, B and D × 400)

Histomorphometric result

There was a highly statistically important rise in the proportion of surface area occupied by fat graft in the study group compared to the control group in all experimental periods of 3, 6, and 9 weeks P=**(<0.001*),** as shown in **Table (1)** comparison between the control and study groups.

As shown in **Table (1)**, both of the control and study groups experienced a statistically noteworthy decline in the percentage of fat graft surface area from 3 weeks to 9 weeks.

Table (1): Comparison between the two studied groups regarding the percentage of surface area of fat graft at 3, 6 and 9 weeks

Percentage of surface area of fat graft	3 weeks (n=5)	6 weeks (n=5)	9 weeks (n=5)	F	p
Control (n=15)					
Min. – Max.	30.10 – 32.50	26.65 – 29.89	17.76 – 20.36		
Mean ± SD.	31.48 ± 1.06	28.09 ± 1.26	18.87 ± 1.0	173.129*	<0.001*
IQR	30.70 – 32.40	27.19 – 28.52	18.39 – 19.32		
Sig. bet. grps	p_1=0.001*, p_2<0.001*, p_3<0.001*				
Study (n=15)					
Min. – Max.	34.97 – 37.33	30.03 – 35.30	25.87 – 29.78		
Mean ± SD.	35.83 ± 1.04	33.14 ± 2.02	27.95 ± 1.66	30.280*	<0.001*
IQR	35.10 – 36.50	32.88 – 34.54	26.92 – 29.46		
Sig. bet. grps	p_1=0.055, p_2<0.001*, p_3=0.001*				
t (p_0)	**13.522* (<0.001*)**	**10.579* (<0.001*)**	**25.714* (<0.001*)**		

IQR: **Inter quartile range** SD: **Standard deviation**

t: Paired t-test

F: **F for One way ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

p_0: p value for **Paired t-test** for comparing between **Control** and **Study**

p: p value for **One way ANOVA test** for comparing between **the two studied groups**

p_1: p value for comparing between **3 weeks** and **6 weeks**

p_2: p value for comparing between **3 weeks** and **9 weeks**

p_3: p value for comparing between **6 weeks** and **9 weeks**

*: Statistically significant at $p \leq 0.05$

DISCUSSION

In the aesthetic and reconstructive surgeries, the autologous fat grafting technique has emerged as a standard procedure for addressing abnormalities of both volume and contour. Breast augmentation, radiation damage, posttraumatic deformities,

congenital defects, and burn injuries have all been treated by applying fat grafting. Earlier studies on fat grafting for facial and breast reconstruction by Neuber, Czerny, and Holländer have revealed the favorable and natural-looking outcomes possible with this procedure (2, 19, 20).

Botulinum toxin type A (BoNTA), which is frequently used for aesthetic operations, effectively reduces facial wrinkles by inducing muscle paralysis. In the early stages of face ageing, loss of soft tissue volume and an increase in wrinkles are common phenomena. Fat grafting has traditionally been used as a treatment (21).

Hence, the target of this experimental research was to test effectiveness of fat graft combined with botulinum toxin in comparison with fat graft alone regarding survival rate on lip of the rabbit model, to assess the area occupied by fat graft in the upper lip histologically and histomorphometrically.

The rabbit model was chosen because of its naturally occurring split "hare lip," which effectively separates the right and left hemilips. This prevents accidental grafting of adipose tissue into the contralateral hemilip and allows for an accurate assessment of what happens to the grafted adipose tissue over time (15).

There are numerous ways to harvest fat; some of the oftenly used in procedures include the direct excision, syringe hand aspiration, and suction-assisted liposuction, which all require different pressures (and also different mechanisms to achieve that pressure) (22-24).

When compared to fresh fatty tissue samples and syringe-aspirated fat, Pu et al. (25, 26) discovered that standard liposuction aspirates severely affected adipocyte activity. However, Qin et al. (25, 27) suggested using the core graft for block grafting because it protects the adipocytes from injury, preserving the structure and viability of the harvested fat tissue.

Although many authors have recommended alternate harvesting methods (and pressures) in an effort to produce improved functional grafts, it is well-acknowledged that great vacuum pressures used in standard liposuction are more detrimental to tissue and can cause adipocyte structural disruption. Direct fat excision over aspiration is sustained by recent experimental research in addition to certain medical ones (25, 28).

Thus, in this study we chose to use fat graft block to maintain the blood supply and the viability of adipocytes and to be able to inject BoNTA to the fat graft as a block to study the survival rate. In contrary, Liposuction traumatize adipocyte structure and endanger their viability.

In the present work, histological results showed that adipocytes were well defined and densely populated in study groups of 3, 6 and 9 weeks. While on the other side, the control groups showed loss in the normal architecture of adipocytes and more degeneration.

According to our Histomorphometric results, the surface area percentage, which is occupied by fat graft in the study group, was also considerably greater than that in the control ($P< 0.001$).

Our findings concur with those of Hoon et al., who discovered that the BoNTA group's adipose tissue volume and weight were significantly larger than those of the control group. Additionally, their histological results indicated that BoNTA can increase the density of blood vessels and adipose tissue while significantly lowering the incidence of adipose tissue fibrosis, which can increase the survival rate of fat transplants (9).

According to this, BoNTA helps fat grafts survive by lowering the lipolysis of transplanted adipose tissues as well as norepinephrine secretion and vasoconstriction. BoNTA is effective at promoting vasodilation due to its lack of impacts on the release of nitric oxide involved in it, which raises the survival rate and nutritional supply (29). Additionally, BoNTA's limitation of muscle activity in the vicinity of the transplanted area can increase the rate of fat retention.

Our results are in line with Baek et al (2012)'s idea that Botulinum Toxin A (BoNTA) improvement of fat grafts could enhance fat graft survival in the facial region. They have investigated this notion in a rat model. According to Baek et al., the volume, weight, and cellular integrity of the graft that received BoNTA were expressively greater than those of the control graft. They found that the initial 0.5 mL of the transplanted fat tissue retained 74% of its volume in the BoNTA group but just 44% in the control (29).

The scientists hypothesised that the greater retention was brought about by the BoNTA's transient muscle immobilization, which lessens abnormal facial muscle spasms and maintains transplant survival (29).

The current study's histology findings revealed that the study group (BoNTA) had more blood vessels than the control (saline). Numerous researches in the adipose tissue engraftment's field have verified the significance of revascularization in the recipient site to escalate the survival of adipose tissue graft (30). According to reports, BoNTA can elevate the skin flap engraftment by re-vascularizing the area (31).

The results of this study are clinically applicable in different cases in oral and maxillofacial surgery, such as, oro-antral fistula closure, pedicle graft after tumor enucleation also used as a membrane for implant. As the main disadvantage of fat graft is being easily resorbed and having low survival rate, so in this study, fat tissue was injected with botulinum toxin to improve the survival rate of fat graft.

CONCLUSION

Adipose tissues, injected with botulinum toxin exhibited a much better and significant

survival rate than fat graft alone. The results of the present study may provide a new idea for new free fat grafting methods in future plastic and reconstructive surgery.

Conflict of interest

The authors declare that they have no conflict of interest.

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