A Pilot Study on Lipolytic Effect of Subcutaneous Botulinum Toxin Injection in Rabbits

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OBJECTIVE: To determine whether botulinum toxin type A (BTX-A) exerts a lipolytic effect by interfering with acetylcholine transmission at the cholinergic parasympathetic nerve endings.

STUDY DESIGN: Fifteen male rabbits were divided into 3 equal groups: 1 control group (A) and 2 case groups (B and C). The abdomens of all rabbits were divided into a 3×3-square grid. The groups received 9 subcutaneous injections of 0.9% normal saline, 1 U BTX-A (group B) and 2 U BTX-A (group C), respectively. Four weeks later the entire grid was excised from the abdominal area. Hematoxylin-eosin–stained tissue was used for stereologic analysis to estimate cell surface and volume in 100 randomly selected cells.

RESULTS: Gross thinning of subcutaneous fat and shattering and disappearance of fat globules were seen in both case groups. Fat cell volume was reduced by 65% in group B (p = 0.009) and 77% in group C (p = 0.009) compared to control animals. Fat cell surface also decreased by 51% in group B (p = 0.009) and 63% in group C rabbits (p = 0.009) compared to control animals.


Keywords: adipose tissue, botulinum toxin, lipolysis.

Today obesity is one of the most problematic health issues, not only in rich countries but also in developing countries. Subcutaneous adiposity leads to many important psychologic disturbances, such as embarrassment, societal and occupational discrimination, lower levels of self-acceptance and consequently impaired quality of life. Available weight-loss therapies include medications such as lipase inhibitors and appetite suppressants, in addition to surgical interventions. Unpleasant side effects and
limited long-term efficacy have made pharmacologic treatment options disappointing. Subcutaneous liposuction causes severe complications, such as thromboembolism, and is associated with mortality rates as high as 1 per 5,000.

The autonomic nervous system plays an important role in controlling fat metabolism in adipose tissue. Parasympathetic input has anabolic effects on adipose tissue by modulating its insulin sensitivity and the metabolism of glucose and free fatty acids, whereas sympathetic stimulations facilitate lipolysis in adipose tissue.

Botulinum toxin (BTX), produced by *Clostridium botulinum*, can interfere with acetylcholine release at the neuromuscular junctions and in the cholinergic parasympathetic and postganglionic sympathetic nervous system. On binding to the cholinergic nerve ending membrane via its heavy chain, the BTX-receptor complex is internalized by endocytosis. The neurotoxin is then translocated to the cytosol to cleave soluble NSF, N-ethyl maleimide-sensitive factor, attachment receptors (SNARE), proteins essential for acetylcholine release.

We investigated the effects of subcutaneous BTX type A (BTX-A) injection on fat cell volume and surface in the abdominal subcutaneous tissue of rabbits using quantitative stereologic methods.

**Materials and Methods**

The current study was conducted in the Comparative Medicine Research Center, affiliated with Shiraz University of Medical Sciences, Shiraz, Southern Iran, from June 2008 to March 2009. Experimental animals were 15 male albino rabbits obtained from the Pasteur Institute of Iran. The rabbits were homogenous in weight (2,500 ± 100 g) and age (6 months). BTX used was Dysport (Beaufour-Ipsen, Dreux, France). The rabbits were divided into 3 equal groups: group A (control group), group B (experimental group in which a 9-mouse unit BTX-A was injected), and group C (experimental group in which an 18-mouse unit BTX-A was injected). Each group contained 5 rabbits. All investigators, including research assistants, pathologists and a statistician, were completely blinded to drug and placebo.

This study was conducted according to the World Medical Association Declaration principles for animal studies, revised in October 2006 and was approved by the Ethics Committee of Shiraz University of Medical Sciences.

The abdomen of all rabbits was divided into a 3×3-square grid (4.5×4.5 cm) of 9 injection sites. The upper injection sites were 2 cm below the diaphragm to avoid diaphragmatic muscle paralysis.

In control group rabbits, 0.01 mL 0.9% normal saline was injected subcutaneously in each site. The vial of 500 U BTX-A was reconstituted with 5 mL of 0.9% normal saline, resulting in a dilution of 1 U of BTX-A in each 0.01 mL. The 2 case group rabbits received 9 subcutaneous injections of 1 U (group B) or 2 U BTX-A (group C). The lethal dose of BTX-A for rabbits is 0.5 ng/kg, which means 10 U/kg. The total injection of 9 and 18 U BTX-A into the abdominal subcutaneous tissue of rabbits was chosen to be under lethal dose for rabbits weighing 2,500 ± 100 g.

Four weeks later, all of the rabbits were anesthetized with ketamine (10% (100 mg/kg) and xylazine 2% (10 mg/kg). The animals’ muscular wall and skin were stitched with chromic and silk sutures, respectively. All of the samples were divided into halves. Half of the specimens were maintained in a fresh state and underwent frozen section at the center of the specimens. They were then stained with oil red O staining. These slides were studied by light microscopy to investigate qualitative changes that happened to the subcutaneous adipose tissue of the rabbits.

The other half of the specimens were fixed in 4% buffered formalin and then cut into small pieces. These parts were randomly oriented and sectioned. We also cut the tissues in random orientations and positions and embedded them randomly in a paraffin block to achieve isotropic uniform sections, as necessary for further stereologic work. Sections with a 10-μm-thickness were obtained. The sections were mounted on slides and stained with hematoxylin-eosin. Microscopic fields were selected in each section in a random manner. The position of the first area was selected randomly outside of the section, and the other areas were selected by moving the microscope stage in an equal interval along the X and Y directions of the stage using a stage micrometer. A high numerical aperture, ×100 magnification (NA = 1.4) oil immersion lens was used. Estimation of fat cell volume and surface was performed at the final magnification of 1,500 using the method described by Cruz-Orive. This method is an estimator of the number-weighted mean particle volume and surface, meaning that cell selection is done according to numerical density in a 3-D space. To do this, the cells must be selected...
with uniform random probability using the “dissector” principle. The optical dissector is a method in which the cells are sampled in thick sections observed with a light microscope. Using the dissector, the particles are selected according to their numerical density, not according to their shape, size and volume. In the sections with 10-μm thickness, the optical section was set at a random depth using a microcator (MT 12, Heidenhain, Traunreut, Germany), which can measure the thickness, or z-axis, of the sections. Briefly, the first 3 μm of the sections were ignored and sampling of the cells was performed in the next 5 μm of the sections. A personal computer and a monitor were connected to a color video camera mounted on top of the microscope. By means of a stereologic software (designed at Shiraz University of Medical Sciences), a test system of quadrangles was superimposed on the images of the tissue sections viewed with a light microscope. Briefly, 100 cells, whose nuclei did not touch the left or inferior borders of each quadrangular frame, were qualified from each rabbit specimen for further estimation of number-weighted mean volume and surface. Each sampled nucleus was adopted as the pivotal point. The uniform random test system of quadrangles with a predetermined area (a) was laid on the image of the cell. Through each pivotal point, a test line (bold lines in Figure 1) was drawn perpendicular to the axis joining the points of the test system to the pivotal point (thin lines in Figure 1). The intercept lengths test lines and the number of intersections (small white circles in Figure 1) with cells was estimated. The equations $V = al$ and $S = 2al$ were used to estimate the volume ($V$) and surface ($S$) of the cells, where $a$ is the area per test point (here 665.64 μm$^2$), $L$ is the sum of the intercept lengths in each sampled cell and $I$ is the sum of the number of intersections of the vertices with the cell borders.

**Statistical Analysis**

The data were compared with the Mann-Whitney U test, and $p < 0.05$ was considered significant.

**Results**

None of the animals developed respiratory depression, and none died during the study. The feeding and daily food intake of both case groups of rabbits were the same as the control group, and we did not observe any differences in daily food intake among groups after injecting case group rabbits with BTX-A. Subcutaneous BTX-A injection resulted in qualitative and quantitative changes in adipose tissue, as reported below.

**Qualitative Changes**

Microscopic evaluation of the specimens underwent frozen section and oil red O staining showed that in abdominal subcutaneous adipose tissue of the BTX-A–treated animals compared with the control group animals, the subcutaneous fat layer became thinner, fat globules became smaller, fragments of fat globules acquired irregular borders, fat globules became shattered compared with the fat globules of the control group and fat globules inside the fat cells disappeared (Figure 2). The difference between the dose of BTX-A injected into the abdominal subcutaneous fat of the rabbits in groups B and C did not produce significant qualitative differences in the changes that occurred in the subcu-
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Quantitative Changes

Fat cell volume was reduced by 65% in group B (p = 0.009) and 77% in group C (p = 0.009) compared to control animals. Fat cell surface also decreased by 51% in group B (p = 0.009) and 63% in group C rabbits (p = 0.009) compared to control animals. Fat cell volume in group C was reduced by 34% compared to group B animals (p = 0.05). The fat cell surface in group C was reduced by 25% compared to group B animals (p = 0.03) (Table I).

Discussion

Our study revealed a dose-dependent lipolytic effect of subcutaneous BTX-A injection in male albino rabbits. Qualitatively, gross thinning of the subcutaneous fat layer and shattering and disappearance of fat globules were seen in both case groups. Injecting BTX-A also induced a statistically significant decrease in the fat cell surface and volume as measured stereologically.

A wealth of evidence reveals that obesity is associated with increased morbidity and mortality.18 Obesity also induces severe psychosocial consequences such as loss of self-confidence and social discrimination.2 Current pharmacologic treatments, such as orlistat and subitramine, and surgical methods, including liposuction and gastric stapling, have their own shortcomings in terms of

Table 1  Mean ± SD of Fat Cell Surfaces and Volumes in the Control and BTX-A–Treated Rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell surface (μm²)</th>
<th>Cell volume (μm³)</th>
</tr>
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<tbody>
<tr>
<td>Control (n = 5)</td>
<td>28,744 ± 2,839</td>
<td>353,684 ± 60,771</td>
</tr>
<tr>
<td>Group B (n = 5)</td>
<td>13,978 ± 3,281ᵃ</td>
<td>123,545 ± 44,533ᵃ</td>
</tr>
</tbody>
</table>
| Group C (n = 5)| 10,434 ± 1,272ᵃ,c | 81,329 ± 14,739ᵃ,b| ⁴

ᵃp ≤ 0.009, BTX-A–treated animals (1 or 2 U at each injection site) vs. control.
ᵇp ≤ 0.05, group C (2 U at each injection site) vs. group B (1 U at each injection site).
ᶜp ≤ 0.03, group C (2 U at each injection site) vs. group B (1 U at each injection site).

Figure 2  Sections from abdominal subcutaneous fat tissue of rabbits. (A) Thinning of fat layer in BTX-A–treated rabbits (right) compared with control (left) in low power light microscopy. (B) Thinning of fat layer in BTX-A–treated rabbits (right) compared with control (left) in high power light microscopy. (C) Small fat globules in BTX-A–treated rabbits (right) compared with control (left). (D) Irregular fragments of fat globules in BTX-A–treated rabbits (right) in comparison with regular round fat globules in control group (left). (E) Shattered fat globules in BTX-A–treated rabbits (right) compared with intact fat globules of control group (left). (F) Disappearance of fat globules inside the fat cell in BTX-A–treated rabbits (right) comparing with the normal fat cell having good fat globules (left) (oil red O, A, × 320; B, C, D, and E, × 900; F, × 1,200).
safety and efficacy.\textsuperscript{4,5,19–21} Tumescent liposuction has been described as a safe procedure but is associated with several complications, such as postoperative pain, syncope, edema, ecchymoses, panniculitis and fat necrosis in diabetic patients, seroma formation and irregularity and asymmetry of the operated area.\textsuperscript{22} The efficacy of mesotherapy, as an alternative to liposuction, has also been questioned, and complications such as bruising, edema, skin necrosis, atypical mycobacterial infections, ecchymoses and hematomas have been reported.\textsuperscript{23,24}

The autonomic nervous system modulates lipolysis and lipogenesis by changing local insulin sensitivity and expression levels of adipokine in adipose tissue and by regulating fat cell numbers.\textsuperscript{6} β-Adrenergic sympathetic fibers have lipolytic action, and parasympathetic fibers have anabolic effects.\textsuperscript{8,25,26}

The injection of BTX causes transient denervation of cholinergic parasympathetic fibers.\textsuperscript{10} This effect has been used to treat sialorrhea.\textsuperscript{27} The lipolytic effect of BTX via inhibition of some parasympathetic anabolic effects was first hypothesized by Lim and Seet.\textsuperscript{28} They postulated that “BTX can be injected into subcutaneous deposits of fat: in the buttocks, thighs or abdominal apron, for cosmetic purposes.”

There is evidence that lipolysis occurs after parasympathetic denervation. In a study in rats, vagotomy was linked to fat degradation in obese animals.\textsuperscript{29} It was also shown that fat pad–specific vagotomy strongly reduced the insulin-dependent uptake of glucose and free fatty acids in adipose tissues, whereas the activity of lipase, a catabolic enzyme sensitive to hormone activity, increased.\textsuperscript{7}

Our animal study provides the first evidence that a lipolytic effect of subcutaneous injections of BTX-A in the abdominal area is “probable rather than fat chance.” This lipolytic effect has 2 implications: (1) a subcutaneous BTX-A injection might be used as a method of melting fat for body sculpting and (2) lipoatrophy may be an undesired side effect of BTX-A injection for cosmetic or therapeutic purposes, especially for facial treatments. It is also important to emphasize that although subcutaneous BTX-A injection can be effective for regional lipolysis, it does not reduce visceral adiposity and therefore cannot decrease the morbidity and mortality induced by obesity.

One of the shortcomings of our study was the small number of animals used, so larger studies in animal models and research are needed in models that more accurately reproduce conditions in humans. The effects of BTX are transitory, lasting for variable periods ranging from 3 months in spastic muscles up to 2 years for sweat glands.\textsuperscript{10,30} Eventually, nerve terminals sprout and new synaptic contacts are formed, so parasympathetic nerve system functioning can be expected to recover after BTX injection.\textsuperscript{9} Nevertheless, the duration of the lipolytic effects of subcutaneous BTX-A injection should be investigated in further studies.

In summary, the potential of subcutaneous BTX-A injection as an effective treatment for abdominal adiposity merits further research.

\textbf{Acknowledgments}

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