BMC Gastroenterology

BioMed Central

Research article

Botulinum toxin in gastric submucosa reduces stimulated HCl production in rats

Matteo Runfola[1], Simone Rossi[1], Simona Panunzi[2], Pier Luigi Spada[1] and Daniele Gui*[1]

Address: [1]Department of Surgery, Catholic University, Rome, Italy and [2]Biomathematics Laboratory, Istituto di Analisi dei Sistemi Informatici, CNR, Rome

Email: Matteo Runfola - runfola@tiscali.it; Simone Rossi - sinodoc@tiscali.it; Simona Panunzi - simona.panunzi@biomatematica.it; Pier Luigi Spada - plspada@hotmail.com; Daniele Gui* - daniele.gui@rm.unicatt.it

* Corresponding author

Published: 10 September 2003

BMC Gastroenterology 2003, **3**:26

Received: 14 March 2003
Accepted: 10 September 2003

This article is available from: http://www.biomedcentral.com/1471-230X/3/26

Abstract

Background: Botulinum toxin blocks acetylcholine release from nerve endings and acts as a long term, reversible inhibitor of muscle contraction as well as of salivary, sweat gland, adrenal and prostatic secretions. The aim of the present study is to investigate whether gastric submucosal injection of botulinum toxin type A reduces stimulated gastric production of HCl.

Methods: Sixty-four rats were randomized in two groups and laparotomized. One group was treated with botulinum toxin-A 10 U by multiple submucosal gastric injections, while the second group was injected with saline. Two weeks later, acid secretion was stimulated by pyloric ligation and acid output was measured. Body weight, food and water intake were also recorded daily.

Results: HCl production after pyloric ligation was found to be significantly lower in botulinum toxin-treated rats (657 ± 90.25 micromol HCl vs. 1247 ± 152. P = 0.0017). Botulinum toxin-treated rats also showed significantly lower food intake and weight gain.

Conclusions: Botulinum toxin type A reduces stimulated gastric acidity. This is likely due either to inhibition of the cholinergic stimulation of gastric parietal cells, or to an action on the myenteric nervous plexuses. Reduction of growth and food intake may reflect both impaired digestion and decreased gastric motility.

Background

Botulinum toxin is produced by the bacterium Clostridium botulinum in seven serotypes (A through G). It is the most powerful known inhibitor of muscular contraction and acts by interfering with acetylcholine release in the neuromuscular plaque [1]. When ingested, botulinum toxin can lead to the form of food poisoning known as botulism; however, it is also a highly effective medication in patients with neural and muscular disorders. When the toxin is injected directly into the muscle, the resultant inhibition of muscle contraction is selective, prolonged and reversible. The effect begins 2–5 days after the injection, reaches its maximum in 7–10 days and lasts 1–2 months depending on the dose. Btx-A binds to a membrane receptor in the cholinergic nerve-ending and enters the fiber, where the active part of the toxin hydrolyses SNAP-25. This protein is a 206-aminoacid hydrophilic protein participating in the SNARE "fusion machine", which binds and merges vescicular and plasma membranes, eventually releasing neuromediators in the

synaptic cleft. By removing a 9 aminoacid residue from SNAP-25, Btx-A determines neuromuscular paralysis. Since 1973, botulinum toxin type A (Btx-A) has been successfully used in the treatment of voluntary muscle contraction disorders, such as strabismus, dystonia, tremors. In particular, in the last decade, clinical studies have described the therapeutic use of Btx-A in some hyperkinetic diseases of the gastrointestinal tract (GIT) [2–4]. Since 1992, the toxin has been successfully used in achalasia patients, particularly poor surgical risk subjects. In 1994 our group described the effect of Btx-A in patients with fissure-in-ano, a disease related to the hypertonus of the internal (smooth) anal sphincter [5,6].

The action of Btx-A is not limited to the neuromuscular junction, as reported in other studies where autonomic neuroglandular transmission was found to be affected. Parotid, submandibular, nasal and sweat glands were shown to reduce consistently their secretion after local injection of the toxin, and the molecule has recently been proposed as a therapeutic option in sialorrhea, in Frey's syndrome and in the hyperhydrosis of the armpit and hand palms [7–12]. Btx-A was also reported to reduce the zymogen secretion from pancreas [13,14].

The motility and the secretory activity of the gastroenteric system depend upon many neuromediators and modulators. Acid secretion is regulated by a cholinergic neuron that causes direct stimulation of the parietal cell [15]. Pyloric ligation powerfully stimulates gastric acid secretion in rats, as demonstrated in well-known experiments [16].

As botulinum toxin selectively inhibits cholinergic neural transmission, we tested the hypothesis that prior treatment with botulinum toxin would reduce the ability of rat stomachs to secrete acid after stimulation.

Methods

Surgical procedure

The experiment was approved by local Ethics Committee. Sixty-four adult Wistar male rats (weight 240–470 g) were randomized into a BTX group (32 rats) and a SALINE group (32 rats). At time 0, anaesthesia was performed with intraperitoneal injections of diazepam (2 mg Kg^{-1}) and ketamine (2 mg Kg^{-1}). Through a midline abdominal incision the stomach was exposed and entered along the large curve. In the BTX group, the submucosa of the gastric fundus and corpus (not the rumen) was injected with Btx-A (Botox; Allergan, Inc., Irvine, CA, U.S.A.) 10 U in 1 mL saline, via four small injections using a 30G needle. The SALINE group was treated with saline in the same fashion. To prevent postoperative dehydration, animals were injected with 5 cc saline subcutaneously on their back. From the first post-operative day (day 1) rats were housed singly in cages and allowed to feed ad libitum (standard dry pellet diet). Body weights, weight of ingested food and milliliters of water intake were measured and recorded every day; on day 13, all rats were starved. On day 14, the rats were anaesthetized, and the pylorus was exposed and ligated through a small laparotomy. The abdominal wall was closed and 5 cc saline were injected subcutaneously. Access to water and food was denied. Four hours later a lethal dose of anaesthetic was given intraperitoneally. Esophagus and duodenum were clipped, the stomach was removed and gastric juice collected. 10 rats (7 BTX and 3 SALINE) died within the course of the two weeks.

Chemical procedure

Collected gastric juice was centrifuged (15 minutes at 4000 rpm). The supernatant was measured and titrated with NaOH to obtain the total acid output as μEq in 4 hours.

Statistics and Modeling

In order to evaluate the significance of the overall difference between the means of the two groups (BTX vs. SALINE), an analysis of variance was conducted on the HCl production of each rat.

The time-course of body weight for each rat was fitted by a piecewise linear regression model: the model assumes a linear trend of body weight decreasing from day 0 until the nadir and increasing thereafter, i.e $w(t) = w_0 + k_d\, t$, $0 = t = t_n$; and $w(t) = w_n + k_g\, (t - t_n)$, $t > t_n$; where w [g] is the weight of the rat as a function of time, w_0 [g] is the initial weight of the rat, w_n [g] is the weight of the rat at the nadir (lowest predicted weight after surgery), k_d [$g\ day^{-1}$] is the rate of weight loss in the first phase, t [day] is the time, t_n [day] is the time needed to reach the nadir weight, k_g [$g\ day^{-1}$] is the rate of weight gain in the second phase. Since $w_n = w_0 + k_d\, t_n$, the model has three free parameters: k_d, k_g and t_n.

Linear regression analysis over time was also performed on observations of time and food and water intake on each rat, to yield estimated values of the model's parameter k_{food} and k_{water} (rate of food and water intake) respectively.

Results

The sample averages, standard deviations and the significance of the difference of HCl production between groups are given in Table 1. A significant difference ($P < 0.002$) results between the two groups, HCl total production being significantly lower in BTX rats.

Rate of body weight loss after surgery was faster, nadir time was later and rate of after-nadir weight gain was slower in BTX treated than in control animals (all

Table 1: BTXvs. SALINE HCl production, body weight time-course parameters, food and water intake rates.

	BTX group	SALINE group	P
HCl production [μEq/4 hrs]	657.031 ± 848.735 (n = 24 *)	1246.645 ± 442.128 (n = 29)	<0.002
basal body-weight [g]	343 ± 51.65 (n = 26)	350 ± 48.95 (n = 29)	N.S.
k_d: rate of weight loss after treatment [g day^{-1}]	-2.326 ± 1.607 (n = 26)	-0.3739 ± 0.8827 (n = 29)	<0.0001
k_g: rate of weight gain after nadir [g day^{-1}]	2.003 ± 1.583 (n = 26)	3.423 ± 1.593 (n = 29)	<0.0025
t_n: days from time 0 to nadir of body weight [day]	2.45 ± 1.13 (n = 26)	1.374 ± 1.382 (n = 29)	<0.004
k_{food}: rate of food intake after treatment [g day^{-1}]	20.016 ± 4.99 (n = 26)	26.881 ± 2.896 (n = 29)	<0.0001
k_{water}: rate of water intake after treatment [g day^{-1}]	38.619 ± 5.325 (n = 26)	51.868 ± 11.378 (n = 29)	<0.05

* Two test tubes broke during centrifugation and datas were not available for HCl measurement.

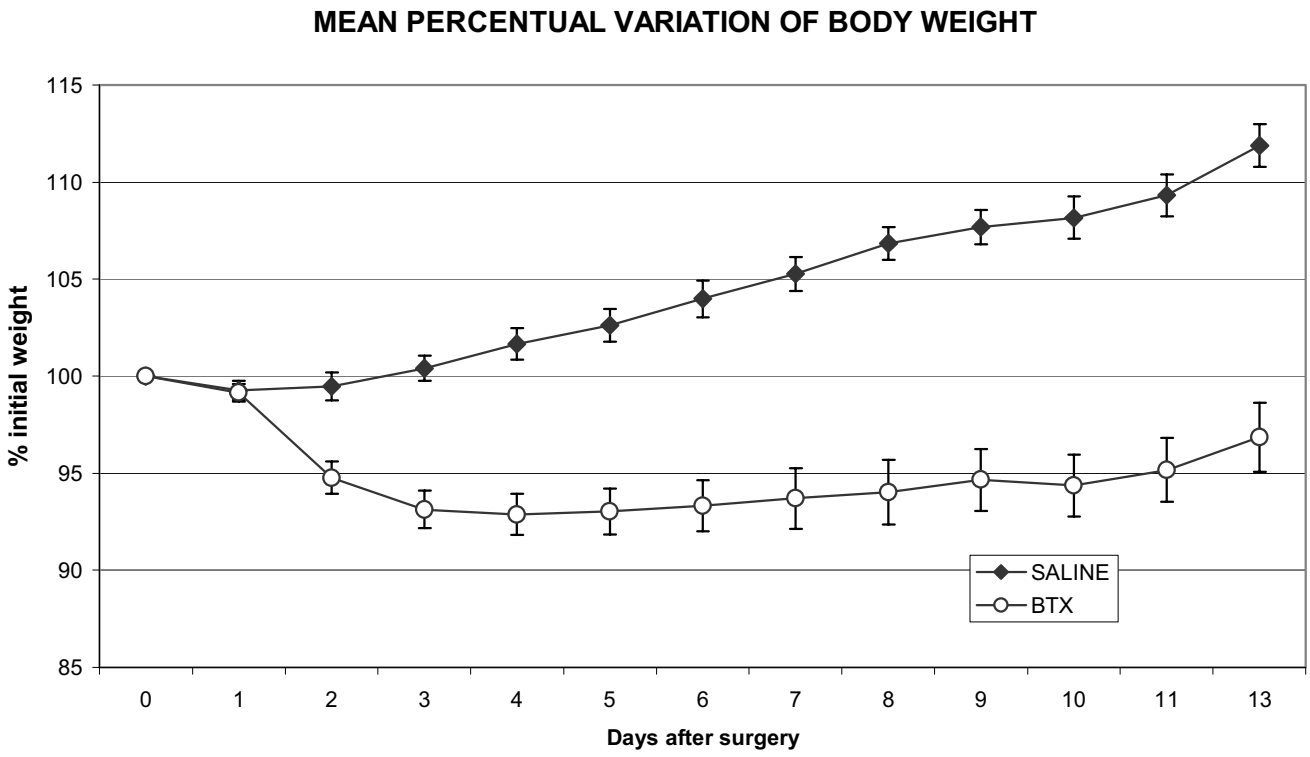

Figure 1
Time course of mean daily percentage variation of body weight in BTX and SALINE groups. Only survivors at day 14 were considered. 100% means basal body weight of each rat.

differences highly significant). Even if rats were not significantly differing in body weight at baseline, the three effects above translate into a depressed weight recuperation of BTX rats after surgery. The average time courses of body weight for both groups are shown in Figure 1. The difference in the time course of body weight was paral-

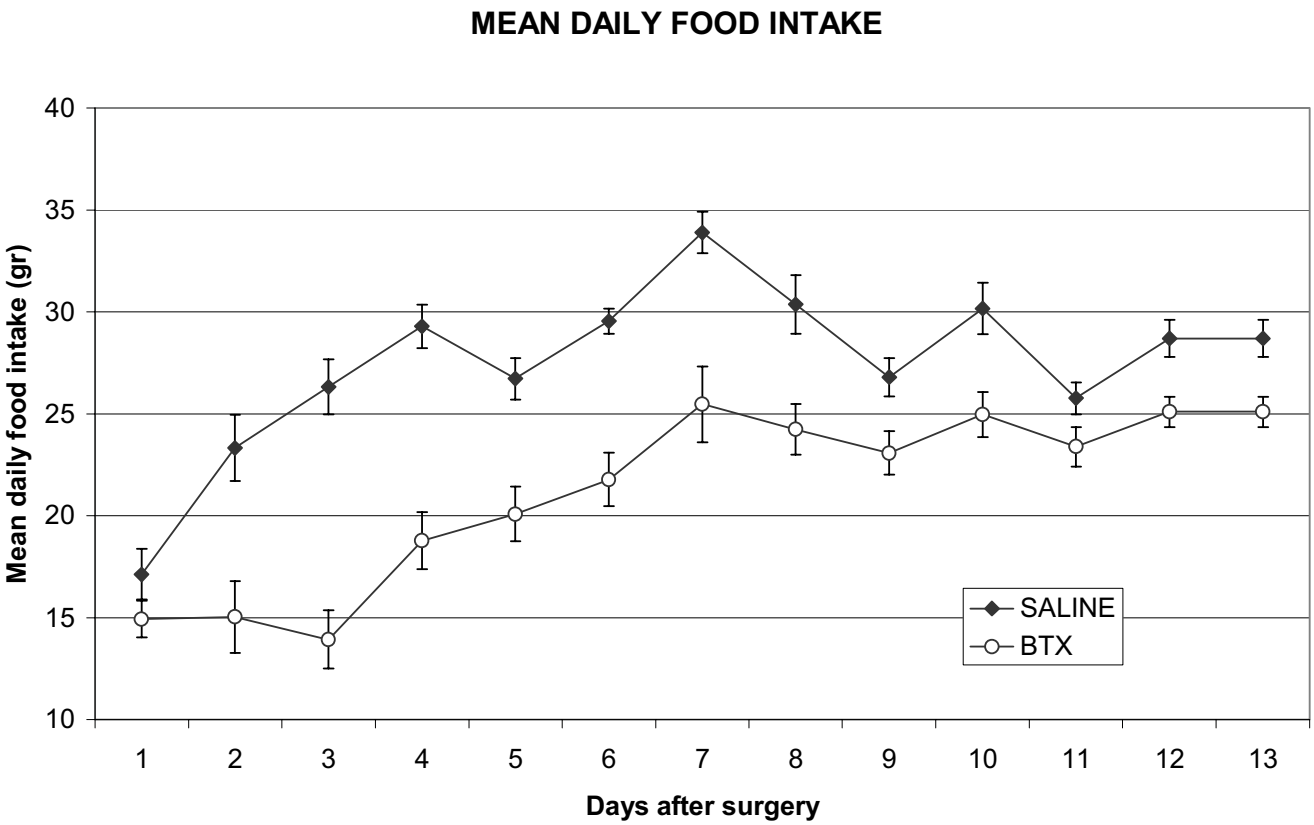

Figure 2
Time course of mean daily food intake in BTX and SALINE groups. Only survivors at day 14 were considered.

leled by a significant difference in food and water consumption rates, Btx-A-treated rats eating and drinking less than control rats. Time courses of food and water intake for both groups are shown in Figures 2 and 3.

Discussion

In the present study, after pre-treatment of experimental animals with Btx-A, the technique of pyloric ligation was adopted. Basically, the mechanism involves vago-vagal reflexes induced by activated mucosal baroceptors in the antrum [17], while non-cholinergic mediators (histamin and gastrin) seem to be less involved [18].

Membrane muscarinic receptors were described on the basolateral plasma membrane of parietal cells, confirming the cholinergic innervation of HCl secretory cells [19,20].

The significant reduction of stimulated acid output in Btx-A treated rats is consistent with the hypothesized block of neuro-glandular transmission in the parietal cells of gastric mucosa. In 1977 Kondo and Magee observed that i.v. botulinum toxin could inhibit the pentagastrin induced and in a lesser extent the histamine induced gastric acid secretion [21]. They also noted that acid response to vagal stimulation was, as expected, abolished while that to methacoline was inaltered. These observation confirm that botulinum toxin doesn't damage the secreting cells, but acts reversibly inhibiting acetylcholine release. Its action is probably so effective on gastric HCl production, because suppressing the cholinergic signal most of the pathways that conduce to acid secretion are all inhibited.

Body weight loss was observed in both groups of rats during the first days after surgery, as a likely consequence of surgical stress. After a few days, SALINE-treated rats were again growing at a normal rate. On the contrary, BTX-treated rats showed persistently lower growth rates, paralleling decreased caloric intake.

The effect is highly reproducible, both in adult and (to a lesser extent) in juvenile animals. Infact in a previous

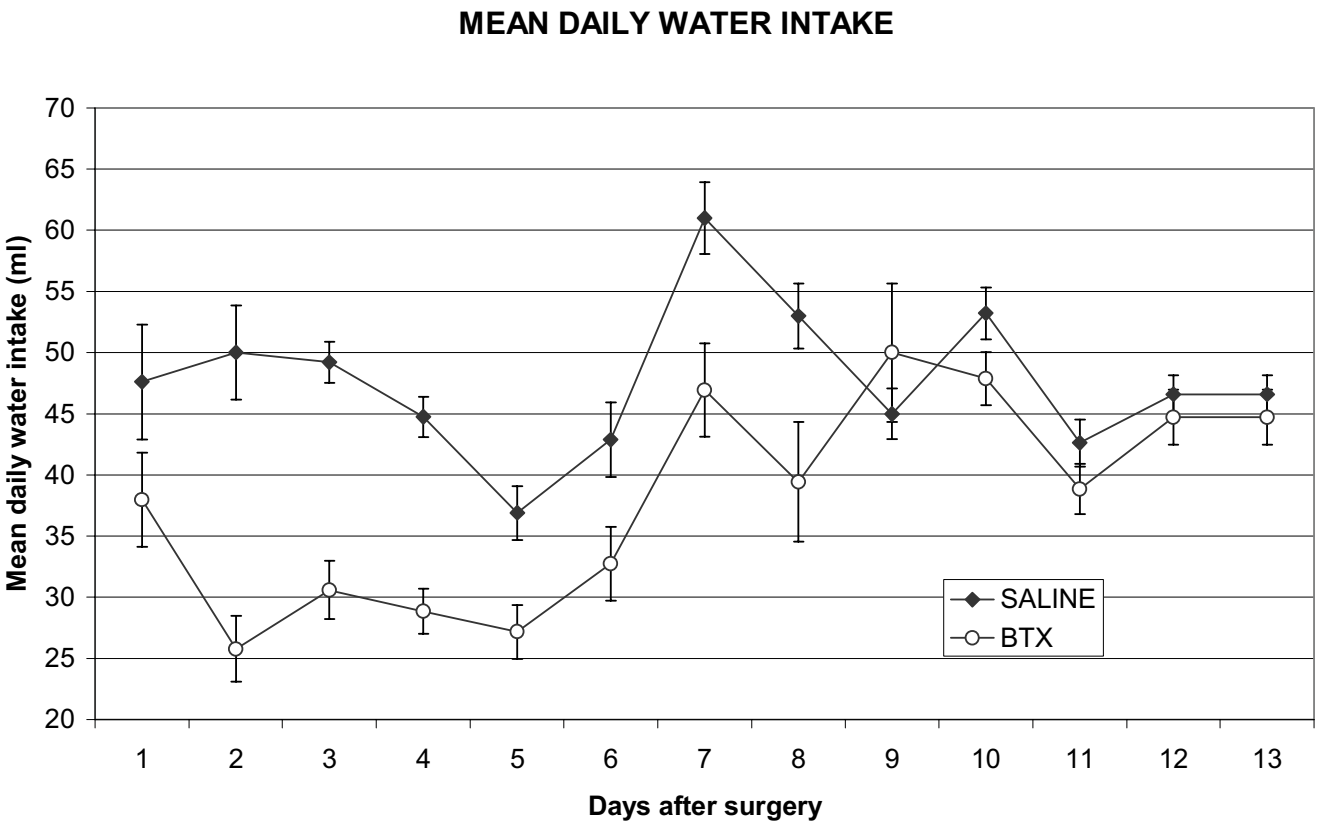

Figure 3
Time course of mean daily water intake in BTX and SALINE groups. Only survivors at day 14 were considered.

study we hypothesized that gastric peristalsis would be impaired and food intake consequently decreased. Some X-rays films of rats fed with contrast showed prolonged gastric emptying times in comparison with non-treated rats [22]. In the present study, the time course of body weight was studied with the aid of a suitable mathematical model, allowing us to quantify a first weight loss phase (which lasts a few days after injection in treated rats) and a second weight gain phase. In the present series, BTX-treated rats had steeper and more prolonged weight loss, as well as slower subsequent weight gain, compared to control animals. The observed effect on body weight might be related to impaired digestive ability, mediated by the inhibition of gastric acid secretion and possibly by other mechanisms.

Conclusions

Btx-A is a potent neuromuscular toxin: when injected in muscle, it reduces the strength of contraction in a dose-dependent manner. The effect is long lasting (1–3 months), reversible, free of major side effects.

The nervous system of the gastrointestinal tract consists of a very rich neuronal network which is organized in gangliar and agangliar plexuses, like the Meissner's and Auerbach's plexuses controlling motility, secretion, absorption and muscolaris mucosae activity.

Since gastric HCl secretion is strongly dependent upon cholinergic activity of vagal and myenteric fibers which ultimately act by their neuroglandular junctions, it was reasonable to suppose it could be inhibited by local injection of Btx-A [23,24]. Gastric acid secretion was induced by the pyloric ligation technique a proven, powerful stimulus of acid secretion. Interestingly, this effect is reduced by vagotomy, vagolytic agents or gangliar blocking agents, i.e. by anti-cholinergic maneuvers. The observed effect on stimulated acid output after submucosal injection of Btx-A is consistent with the hypothesized impairment of neuro-glandular cholinergic transmission. It is also possible that neural stimulation was impaired at the level of myenteric plexuses, where neuro-neuronal synapses are abundant. The injection of toxin was strictly submucosal,

but, since the molecule has diffusive properties, the involvement of fibers of the plexuses cannot be excluded, at least with regards to the internal plexus.

Further studies are warranted to define the site of action of Btx-A in the gastric neural network, and consequently explain the observed effects. However, the present work demonstrates that, whatever the ultimate cause of the weight loss, mucosal Btx-A injection significantly affects the mechanism of gastric acid production and determines prolonged weight loss in experimental animals.

Competing interests
None declared.

Author's contributions
MR, SR, PLS, participated in the design and coordination of the study, performed the surgical procedure and drafted the manuscript.

SP, performed the statistical analysis.

DG, conceived of the study and participated in its design and coordination.

All authors read and approved the final manuscript.

Acknowledgements
This work was supported in part by a Telethon-Italia grant.

References
1. Das Gupta BR: **Structure and biological activity of botulinum neurotoxin.** *J Physiol (Paris)* 1990, **84:**220-228.
2. Jankovic J and Brin MF: **Therapeutic uses of botulinum toxin.** *N Engl J Med* 1991, **324:**1186-1194.
3. Gui D, Rossi S, Runfola M and Magalini SC: **Botulinum toxin in the therapy of gastrointestinal motility disorders.** *Aliment Pharmacol Ther* 2003, **18:**1-16.
4. Maria G, Cassetta E, Gui D, Brisinda G, Bentivoglio AR and Albanese A: **Botulinum toxin for chronic anal fissure.** *N Engl J Med* 1998, **338:**217-220.
5. Brisinda G, Maria G, Bentivoglio AR, Cassetta E, Gui D and Albanese A: **A comparison of injection of botulinum toxin and topical nitroglycerin ointment for the tratment of chronic anal fissure.** *N Engl J Med* 1999, **341:**65-69.
6. Gui D, Cassetta E, Anastasio G, Bentivoglio AR, Maria G and Albanese A: **Botulinum toxin for chronic anal fissure.** *Lancet* 1994, **344:**1127-1128.
7. Shaari CM, Wu BL, Biller HF, Chuang SK and Sanders I: **Botulinum toxin decreases salivation from canine submandibular glands.** *Otolaringol Head Neck Surg* 1998, **118:**452-457.
8. Bushara KO: **Sialorrhea in amyotrophic lateral sclerosis: a hypotesis of a new treatment – botulinum toxin A injections of the parotid glands.** *Med Hypoteses* 1997, **48:**337-339.
9. Bjerkhoel A and Trobbe O: **Frey's syndrome: treatment with Botulinum toxin.** *Laryngot Otol* 1997, **111:**839-844.
10. Dulguerov P, Quinodoz D, Cosendai G, Piletta P and Lehmann W: **Frey syndrome treatment with botulinum toxin.** *Otolaryngol Head Neck Surg* 2002, **122:**821-827.
11. Solomon BA and Hayman R: **Botulinum toxin type A therapy for palmar and digital hyperhidrosis.** *J Am Acad Dermatol* 2000, **42:**1026-1029.
12. Naver H, Swartling C and Aquilonius SM: **Palmar and axillary hyperhidrosis treated with botulinum toxin: one-year clinical follow-up.** *Eur J Neurol* 2000, **7:**55-56.
13. Sadoul K, Lang J, Montecucco C, Weller U, Regazzi R, Catsicas S, Wollheim CB and Halban PA: **SNAP-25 is expressed in islets of Langerhans and is involved in insulin release.** *J Cell Biol* 1995, **128:**1019-1028.
14. Wheeler MB: **Characterization of SNARE protein expression in beta cell lines and pancreatic islets.** *Endocrinology* 1996, **137:**1340-1348.
15. Schubert ML and Shamburek RD: **Control of acid secretion.** *Gastroenterol Clin North Am* 1990, **19:**1-25.
16. Shay H, Komarov SA, Fels D., Meranze D., Gruenstein H. and Siplet H: **A simple method for the uniform production of gastric ulceration in the rat.** *Gastroenterology* 1945, **5:**43-61.
17. Brodie DA: **The mechanism of gastric hyperacidity produced by pylorus ligation in the rat.** *Am J Dig Dis* 1966, **11:**231-244.
18. Alumets J, Ekelund M, Hakanson R, Hedenbro J, Rehfeld JF, Sundler F and Vallgren S: **Gastric acid response to pylorus ligation in rats: is gastrin or histamine involved?** *J Physiol* 1982, **323:**145-156.
19. Nakamura M, Oda M, Yonei Y, Tsukada N, Komatsu H, Kaneko K and Tsuchiya M: **Muscarinic acetylcholine receptors in rat gastric mucosa. A radioautographic study using a potent muscarinic antagonist, 3H-pirenzepine.** *Histochemistry* 1985, **83:**479-487.
20. Tokunaga T, Nishimura R and Akagi M: **Muscarinic cholinergic receptors in human gastric mucosa.** *Jpn J Surg* 1984, **14:**122-126.
21. Kondo T and Magee DF: **Modification of the action of pentagastrin on acid secretion by botulinum toxin.** *Experientia* 1977, **33:**750-1.
22. Gui D, De Gaetano A, Spada PL, Viggiano A, Cassetta E and Albanese A: **Botulinum Toxin injected in gastric wall reduces body weight and food intake in rats.** *Aliment pharmacol Ther* 2000, **14:**834.
23. Ekblad E, Mei Q and Sundler F: **Innervation of the gastric mucosa.** *Microsc Res Tech* 2000, **48:**241-257.
24. Lechago J and Barajas L: **Cholinergic innervation of the simian oxyntic gastric mucosa.** *Am J Anat* 1976, **147:**147-152.

Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-230X/3/26/prepub