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NEUROLOGY AND PRECLINICAL NEUROLOGICAL STUDIES - ORIGINAL ARTICLE

Botulinum toxin type D blocks autonomic cholinergic synapses in humans: discussion of a potential therapeutic use

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Abstract
Based on epidemiological data it was believed that botulinumtoxin type D (BT-D) may not block human cholinergic synapses. We wanted to investigate BT-D's effect on the autonomic cholinergic synapse in humans. For this, we compared in four volunteers intraindividually the hypohidrotic effect of intradermal BT-D and BT-A in Minor's iodine starch sweat test. Altogether, we studied BT-D in doses of 4, 8, 16 and 32MU and BT-A in doses of 2, 4, 8 and 16MU at weekly intervals throughout a period of 13 weeks. All BT doses were diluted in 0.2 ml 0.9% NaCl/H_2O. Overall 704 data points were collected. Combined over all four subjects and all four doses BT-D's hypohidrotic effect intensity was half of BT-A's. BT-D's effect peaked around 5 weeks, BT-A's around 7 weeks. BT-D's effect duration was around 12 weeks, of BT-A's was around 14 weeks. For both BT types the hypohidrotic effect was dose dependent. BT-D, when injected intradermally, can block autonomic cholinergic synapses in humans. Compared to BT-A, BT-D's effect intensity was half and its effect duration was some 2 weeks shorter. With its weaker and shorter effect BT-D does not seem to promise therapeutic effects superior to BT-A.

Keywords Botulinum toxin type D · Sweating · Autonomic synapse · Cholinergic synapse · Minor test

Introduction

Botulinum toxin (BT) exists in 7 serotypes (BT-A to BT-G) and numerous additional isoforms. BT can block neuromuscular cholinergic synapses and autonomic cholinergic synapses. Based on epidemiological data it was believed that BT-D (Demarchi et al. 1958; see introduction of Pellett et al. 2015)—and BT-C (Oguma et al. 1990)—may not be able to block human cholinergic synapses. After it was shown that BT-D (Eleopra et al. 2013) and BT-C (Eleopra et al. 1997) do, indeed, block neuromuscular cholinergic synapses in humans when injected intramuscularly, we investigated whether intracutaneous BT-D is also able to block autonomic cholinergic synapses in humans. BT-D's effects, as outlined in this study, will be discussed with respect to its potential therapeutic use.

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Methods

Design

The study followed an intraindividual head-to-head comparison of the hypohidrotic effect of 4, 8, 16 and 32 MU BT-D and of 2, 4, 8 and 16MU BT-A in Minor's iodine starch sweat test. The study was approved by the local ethics committee and performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Botulinum toxin

BT-A used in these experiments was incobotulinumtoxinA (INCO, Xeomin®, Merz Pharmaceuticals, Frankfurt/M, Germany). BT-A was reconstituted with 0.9% NaCl/H_2O to

produce different dilutions containing 2, 4, 8 and 16MU per 0.2 ml. BT-D was produced by the Institute of Pharmacology and Toxicology of Hannover Medical School. BT-D was diluted with 0.9% $NaCl/H_2O$ to produce different dilutions containing 4, 8, 16 and 32MU per 0.2 ml.

Application

Each subject received 4 BT-A injections with 2, 4, 8 and 16MU on one side of the abdominal skin and 4 BT-D injections with 4, 8, 16 and 32MU BT-D on the contralateral side of the abdominal skin. Each injection was placed intracutaneously using a 0.40 × 12 mm injection needle. The injection site was compressed for 10s to reduce BT washout and to facilitate BT tissue diffusion.

Subjects

Altogether four healthy volunteers (4 males, age 41.0 ± 8.3 years) participated in the experiments. All volunteers are co-authors of this publication.

Sweat test

Sweating was induced by increased ambient temperature in a sauna at 90 °C until sufficient sweating occurred. Hypohidrotic areas were determined using Minor's iodine starch test (Minor 1928). Then a transparent foil was placed onto the skin and the outer circumference of the iodine-coloured hypohidrotic area was marked with a pen. Subsequently, the transparent foil was transferred onto millimetre paper and the hypohidrotic area was planimetered by hand. Hypohidrotic areas are given in mm^2. Planimetric data were used to calculate hypohidrotic areas for each subject, for each BT type, each BT amount applied and for each measurement time. Raw data were further analysed by calculations of various mean values and standard deviations. Significance level was set to $p = 0.05$.

Results

Altogether 8 injections in 4 volunteers were performed in weekly intervals during a period of 13 weeks so that 22 × 8 × 4 (= 704) data points were collected throughout the study.

Figure 1 shows an original photo of the hypohidrotic area produced by application of BT-D 16MU. Within the hypohidrotic area sweating was completely blocked. There was a relatively clear margin towards the unaffected skin. When all data points (all BT doses, all measurement times, all subjects) were compared the grand average of BT-A was 230.0 ± 160.1 mm^2 and of BT-D was 245.4 ± 153.5 mm^2

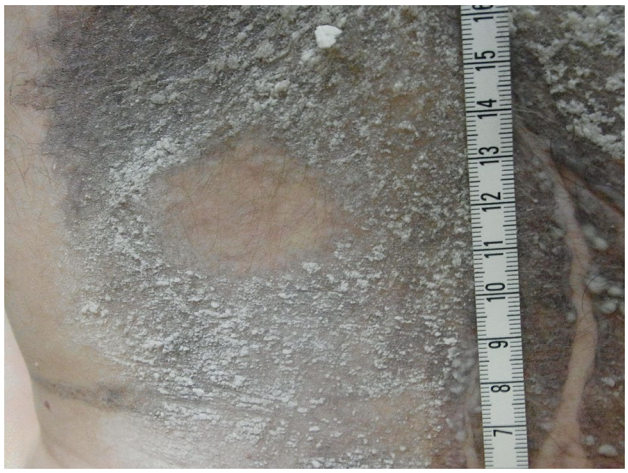
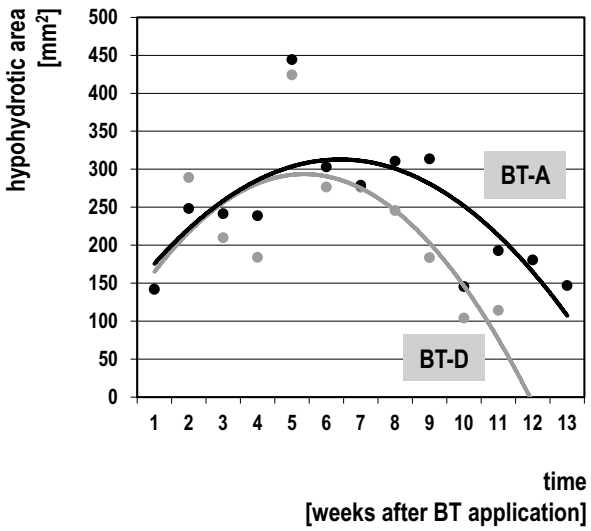


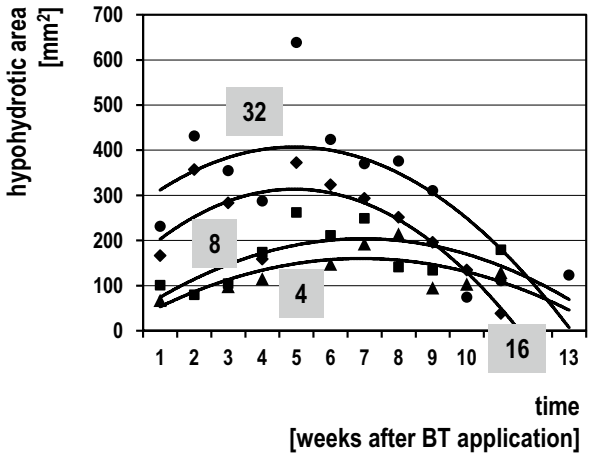
Fig. 1 Hypohidrotic area produced by intracutaneous application of 16MU botulinum toxin type D. Raw data



BT-A: Botulinum toxin type A
BT-D: Botulinum toxin type D
Polynomic trendlines

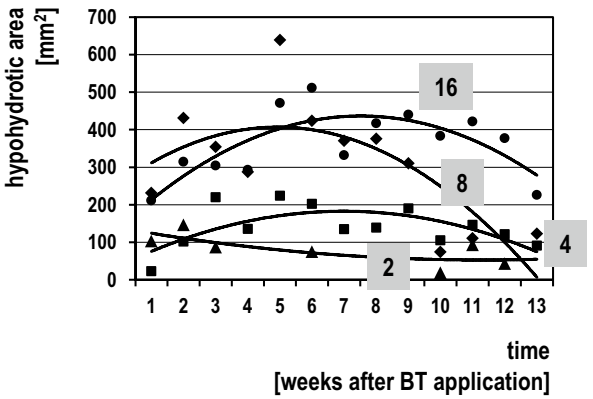
Fig. 2 Hypohidrotic effects produced by intracutaneous application of botulinum toxins types D and A and measured by hypohidrotic skin size. Each data point represents the values of all four subjects and all four dosages ($n = 16$)

(mean standard ± deviation) (Mann–Whitney U test for non-normal distribution, $p = 0.39$). Figure 2 shows the size of the hypohidrotic area of all four volunteers and all four different doses for application of BT-D and of BT-A. BT-D doses of 4MU, 8MU, 16MU and 32MU produce similar hypohidrotic areas as BT-A doses of 2MU, 4MU, 8MU and 16MU. The duration of effect of BT-D is around 12 weeks whereas it is around 14 weeks for BT-A. Figure 3 shows the size



4: Botulinum toxin type D 4MU
8: Botulinum toxin type D 8MU
16: Botulinum toxin type D 16MU
32: Botulinum toxin type D 32MU
polynomic trendlines

Fig. 3 Hypohidrotic effects produced by intracutaneous application of botulinum toxin type D and measured by hypohidrotic skin size. Each data point represents the mean values ($n=4$) of all four subjects



2: Botulinum toxin type A 2MU
4: Botulinum toxin type A 4MU
8: Botulinum toxin type A 8MU
16: Botulinum toxin type A 16MU
polynomic trendlines

Fig. 4 Hypohidrotic effects produced by intracutaneous application of botulinum toxin type A and measured by hypohidrotic skin size. Each data point represents the values of all four subjects ($n=4$)

of the hypohidrotic area of all four volunteers for all four BT-D doses applied. Each data point represents the mean of four values. The size of the hypohidrotic area is correlated to the BT-D dose applied. A dose–duration correlation is unclear. Figure 4 shows the size of the hypohidrotic area of all four volunteers for all four BT-A doses applied. Each data point represents the mean of four values. The size of the hypohidrotic area is less clearly correlated to the BT-A dose applied. A dose–duration correlation is also unclear.

Discussion

Basic mechanisms of BT action

Our data show—for the first time—that BT-D can block autonomic cholinergic synapses innervating the human sweat gland thus producing hypohidrosis when injected intradermally. This is in line with previous observations that application of BT-D (Eleopra et al. 2013) and BT-C (Eleopra et al. 1997) can produce neuromuscular blockade and paresis when they are injected intramuscularly. The maximal BT effect is non-distinguishable between BT-D and BT-A when BT-D is given in doubled doses, i.e. the effect size of BT-D is half of the effect size of BT-A, when identical doses are compared. Lack of peroral activity of BT-D and BT-C may be caused by insufficient gastrointestinal absorption (Simpson 2004), whereas bypassing gastrointestinal absorption by direct injection into the target tissues is able to elicit typical BT effects.

In our model BT-D's and BT-A's effect show a dose–effect correlation. BT-D's effect intensity is half of the effect intensity of BT-A. BT-D's and BT-A's effect duration shows a somewhat loose (and not statistically significant) dose–duration correlation. In order to determine BT-D's effect duration both compound's effect intensities have to be matched and adjusted. When this was done as shown in Fig. 2, where BT-D doses were compared to halved BT-A doses, BT-D's effect duration seems to be some 12 weeks and BT-A's effect duration seems to be some 14 weeks. Likewise, when BT-D 16MU was matched to BT-A 8MU as shown in Figs. 3 and 4, BT-D's effect duration seems to be around 11 weeks and BT-A's seemed to be around 12 weeks. Altogether, BT-D seems to have an effect duration reduced by some 10% compared to BT-A.

Suitability of BT-D for therapeutic purposes

When different BT serotypes are monitored for therapeutic suitability profiling of candidate serotypes along four axes is necessary. These axes are effect intensity (axis 1), effect duration (axis 2), differential efficacy on neuromuscular and autonomic transmission (axis 3) and antigenicity (axis 4). They will be discussed in the following for the BT-B drug rimabotulinumtoxinB and then for BT-D.

For BT-B, effect intensity on axis 1 is 40 times smaller than that of BT-A on axis 1 (Dressler and Bigalke 2009), whereas effect duration on axis 2 seems to be similar

(Dressler and Bigalke 2009). On axis 3 there are major differences between BT-A and BT-B. Whereas BT-A exerts a robust effect intensity on the cholinergic neuromuscular synapse and a relatively weak effect intensity on the cholinergic autonomic synapse, BT-B's profile seems to be reversed (Dressler and Benecke 2003). On axis 4 substantial antigenicity was one of the reasons preventing drug rimabotulinumtoxinB's wide-spread use (Dressler et al. 2003; Dressler and Bigalke 2004).

For BT-D, our study reveals a 50% reduced effect intensity on axis 1 and a mildly reduced effect duration on the autonomic cholinergic synapse on axis 2. BT-D's effect on the neuromuscular cholinergic synapsis has not been outlined so far. It was only felt that BT-D may have an 'overall weak effect' (Eleopra et al. 2013). Subsequently, a differentiation between effect intensity on axis 1 and effect duration on axis 2 could not be obtained. Antigenicity on axis 4 is not known so far as there are no animal studies and no human long-term studies available. However, reduced potency of BT-D and, therefore, an increased protein load may predict unfavourable antigenicity features.

Outlook

More detailed exploration of BT-D's neuromuscular effects in the extensor digitorum brevis test (Kessler and Benecke 1997) or the sternocleidomastoideus test (Dressler et al. 2000) seems to be necessary together with a subsequent antigenicity testing before BT-D might be considered for therapeutic purposes.

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Compliance with ethical standards

Conflict of interest DD received honoraria for services provided to Allergan, Ipsen, Merz, Lanzhou Institute of Biological Products, MedyTox, Revance, Desitin, Syntaxin, Abbvie, Medtronic, St Jude, Boston Scientific, Almirall, Bayer, Sun, Teva, UCB, IAB-Interdisciplinary Working Group for Movement Disorders. He is shareholder of Allergan and holds patents on botulinum toxin and botulinum toxin therapy. KK received travel grants and honoraria for lectures from Allergan, Biogen, Ipsen and Merz. TK used to be member of the advisory board of Allergan (04/12-09/14). He has filed a patent on the use of botulinum toxin in the treatment of personality disorders. He received honoraria for talks from Allergan, Lilly, Lundbeck, Novartis, Otsuka, Schwabe, Servier and Trommsdorf for talks and advisory board activities. NG has no conflicts of interest. SS has no conflicts of interest. HB has no conflicts of interest.

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