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Fund for the Replacement of Animals in Medical Experiments (FRAME)

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LD₅₀ Testing of Botulinum Toxin for Use as a Cosmetic

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Summary

In 2003, the Fund for the Replacement of Animals in Medical Experiments (FRAME) revealed that the potency of botulinum toxin for use as a popular wrinkle treatment is assessed by using the LD₅₀ Test. The endpoint in this mouse-based testing is death through suffocation. In 2004, The Humane Society of the United States (HSUS) sought to work with Allergan, the US-based manufacturer of Botox® Cosmetic, and the U.S. Food and Drug Administration (FDA), on ways to refine, reduce, and replace this LD₅₀ testing. This article summarises The HSUS's campaign in the United States and provides an update on FRAME's continuing efforts in Europe.

Keywords: Allergan, LD₅₀, Botox, botulinum toxin, Dysport, FDA, potency

Introduction

Botulinum toxin, produced by various bacteria, including *Clostridium botulinum*, is one of the most powerful biological toxins known, which blocks transmission of nerve impulses to muscles. Eight subspecies of the bacterium produce seven distinct types of toxin (types A-G), which act through different mechanisms. Food-borne botulinum toxin causes Botulism.

Several companies use botulinum toxin as the active ingredient in therapeutic products for treatment of conditions such as cervical dystonia, strabismus, blepharospasm, and hyperhidrosis. For example, Ipsen Limited UK markets a botulinum toxin Type A product, Dysport®, while the Allergan Corporation, based in the United States, markets two botulinum toxin Type A products: Botox®, for therapeutic applications, and Botox® Cosmetic, the popular wrinkle treatment – a cosmetic application.

Botulinum toxin is produced for commercial application in fermentation batches seeded with the bacteria. The standard method for assessing the potency of botulinum toxin batches is a mouse LD₅₀ Test (Bottrill, 2003). In this procedure, mice are sorted into dose groups, given a single injection of toxin, and monitored over 3-4 days. Death is the endpoint, which results from suffocation through paralysis of the diaphragm musculature. Although the precise details are not available, over 100 mice are used per test, and the mouse testing is carried out up to three times prior to batch release. Calculations from the test data yield an LD₅₀ value (the dose which would kill half the number of animals in a test group), which is then standardised as one “mouse unit”.

The LD₅₀ testing of botulinum toxin products runs counter to three trends in the application of the Three Rs of replacement, reduction, and refinement, and in animal welfare generally. First, the use of the LD₅₀ test is being phased out worldwide. This was symbolised most dramatically in the field of industrial chemicals, when, in 2002, the Organization for Economic Cooperation and Development deleted the LD₅₀ Test (its Test Guideline 401) from its Health Effects Test Guidelines (OECD, 2002). Second,

the use of death as an endpoint is the *bête noire* of the growing field of humane endpoints (Olfert et al., 1998; OECD, 2000; ILAR, 2000). The third trend, applicable to LD₅₀ testing of Botox Cosmetic, is the phasing out of animal testing of products with a cosmetic use. For example, in 2004, the European Union banned all forms of animal testing of cosmetic products (Europa, 2003).

In 2003, the Fund for the Replacement of Animals in Medical Experiments (FRAME) drew attention to the issue of LD₅₀ testing of botulinum toxin products with the publication of an exposé entitled “Growing Old Disgracefully ...” (Bottrill, 2003; Balls, 2003). The Humane Society of the United States (HSUS) then took up the challenge in the USA. FRAME focuses its efforts on the European scene, and The HSUS on the US scene, but we are pleased to have this opportunity to show that we work together, and to provide brief updates on earlier assessments of alternatives to the mouse LD₅₀ testing of botulinum products (Bottrill, 2003; Balls, 2003) and on the FRAME campaign, as well as a summary of the HSUS campaign.

Background

An update on alternatives for the potency assessment of botulinum toxin products

Table 1 summarises some of the potential alternatives to the mouse LD₅₀ Test for assessing the potency of botulinum toxin products. Much of this information is taken from Bottrill's 2003 review. Potential refinements include mouse-based methods that assess local paralysis either *in vivo* or *ex vivo*, in contrast to systemic paralysis *in vivo*, as in the LD₅₀ assay. Potential replacements target the specific molecules involved in nerve transmission that are disrupted by the various types of botulinum. For example, the SNAP-25/endopeptidase assay (Ekong et al., 1997) assesses *in vitro* the extent to which botulinum toxin Type A disrupts the activity of synaptosomal-associated protein of molecular mass 25kDa (SNAP-25), a molecule with a critical role in transmitting nerve signals.



In their recent monograph on testing botulinum toxin, the influential European Pharmacopeia (Anon., 2005) recognised the potential of these methods to substitute for the mouse LD₅₀ test, stating that: “After validation with respect to the LD₅₀ assay (reference method), the product may also be assayed by other methods that are preferable in terms of animal welfare”, including the *in vitro* endopeptidase assay, the *ex vivo* assay using the mouse phrenic nerve diaphragm, and the “mouse bioassay using paralysis as the endpoint.”

The European Pharmacopeia monograph was published prior to the publication of the Endopep-MS assay *in vitro* method (tab. 1), which has the potential advantage of permitting assessments of the potencies of all of the types of botulinum toxin.

An update on the FRAME campaign

FRAME is concerned that Ipsen Limited UK continues to use the mouse LD₅₀ test to measure the potency of Dysport, and urges the Home Office (the British Government department responsible for the control of animal experimentation) to do more to bring about an end to the animal testing of botulinum toxin products for clinical and/or cosmetic use, and considers that the Government should close the loophole which permits botulinum toxin destined to be used for cosmetic purposes to be tested in animals, despite the ban on testing cosmetic products in the UK. The claim is that botulinum toxin is only officially licensed (and therefore tested) for clinical use, and its use for cosmetic purposes involves a private contract between a physician and a patient, at their own risk.

FRAME applauds the efforts of the UK national control agency, the National Institute for Biological Standards and Control (NIBSC), to develop and use refinement and replacement alternatives. The NIBSC uses *in vitro* methods on a routine basis, and only uses a non-lethal *in vivo* test when, rarely, the results of an *in vitro* test are inconclusive or close to pass/fail specifications.

FRAME is also encouraged by the effort being put by the NIBSC into the development of methods which could totally obviate the need for animal testing (eg, Ekong et al., 1997), and also that Ipsen Limited UK are working with the NIBSC and others to develop suitable batch release tests.

Meanwhile, at the European level, the European Centre for the Validation of Alternative Methods (ECVAM) and the European Directorate for the Quality of Medicines (EDQM) are working together and with others to review what progress is being made in applying the Three Rs to botulinum toxin testing and to assist in moving forward.

Summary of HSUS campaign

The HSUS campaign focuses exclusively on the testing of Botox Cosmetic by its manufacturer, Allergan, Inc., based in California. Botox Cosmetic wrinkle treatment is the most common cosmetic procedure in United States, with 2.8 million treatments carried out in 2004 (Allergan, 2005; ASAPS, 2005), accounting for 40% of net Botox sales or \$295M. The HSUS regards the LD₅₀ testing of products for cosmetic use as unacceptable, and seeks to hold Allergan accountable.

The strategy was to first seek to work *with* Allergan, and only if that approach failed, would The HSUS seek to pressure the company from the outside. Three things were sought from Allergan:

1. public disclosure of the details of its current potency testing of Botox Cosmetic;
2. public disclosure of the details of its current efforts to develop alternatives to the mouse LD₅₀ testing of Botox Cosmetic; and
3. adoption of a well-funded and publicly available plan to rapidly end the LD₅₀ testing of Botox Cosmetic.

For several months, beginning in January 2004, The HSUS engaged in cordial, but largely fruitless, communication with Allergan. The company communicated with The HSUS only through its legal staff. At The HSUS's request, the company met with Alan Goldberg, Director of the Johns Hopkins Center for Alternatives to Animal Testing (CAAT), to discuss potential CAAT/Allergan collaboration on alternatives, but the company never followed up this suggestion.

Allergan did confirm that the company uses the LD₅₀ Test to assess the potency of Botox Cosmetic, and claimed to have an active alternatives program to replace this testing. However, the company provided few details either of its current testing practices or of its alternatives efforts. In its defense, Allergan noted that Botox Cosmetic and its sister product, Botox, share the same active ingredient, botulinum toxin Type A, so LD₅₀ testing for the two products is inextricably linked and testing for cosmetic purposes cannot be cleanly separated from testing for therapeutic purposes. Allergan also noted the international regulations calling for the LD₅₀ testing of botulinum toxin products.

The HSUS took note of these claims, but concluded that they collectively failed to justify the company's secrecy concerning its testing and alternatives practices. In The HSUS's view, any company that is making \$300M a year on the backs of suffocating animals deserves to be held publicly accountable for working towards an urgent solution, especially in the context of a cosmetic application.

Tab. 1: Promising alternative methods to the mouse LD₅₀ test for assessing the potency of botulinum toxin products

Name of Test	System	Endpoint	Duration	Reference
Mouse hind-limb assay	<i>in vivo</i>	local paralysis	2 days	Pearce et al., 1995
Abdominal ptosis assay	<i>in vivo</i>	local paralysis	< 1 day	Takahashi et al., 1990
Mouse phrenic nerve-hemidiaphragm system	<i>ex vivo</i>	muscle contraction	< 1 day	Bigalke et al., 2001
SNAP- 25/Endopeptidase assay	<i>in vitro</i>	molecular disruption of nerve transmission	< 1 day	Ekong et al., 1997
Endopep-MS	<i>in vitro</i>	molecular disruption of nerve transmission	< 1 day	Boyer et al., 2005



Consequently, The HSUS decided to implement the second, conditional part of its strategy towards Allergan, namely, applying public pressure to the company. Beginning in October 2004, The HSUS began issuing calls to its members and constituents to urge the company to work with The HSUS on rapidly replacing LD₅₀ testing for Botox Cosmetic. The calls were issued through the organisation's electronic and hard-copy publications. In response to one appeal, thousands of e-mails to Allergan compelled the company to shut down an e-mail account.

Since Allergan refused to work with The HSUS or to disclose information about its testing and alternatives practices, The HSUS turned to the U.S. Food and Drug Administration (FDA), which had approved Botox Cosmetic and Botox. The FDA regulates these products as pharmaceuticals, and now oversees their manufacture and sale. The HSUS was specifically interested in information about the potency testing currently required or encouraged for these products. It was hoped that the agency could help answer several key questions, including the following.

1. What are the current testing practices?
2. Does the FDA require or encourage these practices?
3. How have these practices changed over the years?
4. What is the FDA itself doing to promote LD₅₀ alternatives?

In 2004, The HSUS filed two Freedom of Information Act requests with the FDA, in order to obtain the sought-after information, but the agency was largely unresponsive. Consequently, the HSUS initiated legal action in 2005 to obtain the requested documents. This legal action is still pending.

Discussion and conclusions

FRAME, The HSUS, and similar organisations engage in advocacy of the Three Rs, because they want to accelerate the pace of progress in the development, validation, and implementation of methods to replace, reduce and refine animal experimentation and testing. In the case of the potency testing of botulinum toxin products, it is clear that some progress on alternative methods had been made prior to the launch of the FRAME and HSUS campaigns. However, it is clear that an unknown, but undoubtedly large number of mice were being used, and are still being used, in painful and lethal procedures for the testing of products destined for use for cosmetic, as well as for clinical, purposes. We are prepared to give Allergan, Ipsen Limited UK, and other manufacturers of botulinum toxin products the benefit of the doubt, by accepting that they are seeking to contribute to progress in the right direction.

Given this concession, some might conclude that our advocacy efforts are misplaced. We would disagree. As outlined above, the LD₅₀ testing of botulinum toxin products in general, and of products for cosmetic use in particular, runs counter to three trends: the phasing out of the LD₅₀ test, of the use of death as an endpoint, and of any animal testing of products with a cosmetic purpose. Consequently, the continuation of such testing is particularly out of step with the times, and is therefore particularly in need of scrutiny and action. Instead of assurances that

progress is being made, what is needed is a demonstration of goodwill and verifiable action on the part of the manufacturers and the agencies responsible for the registration and use of pharmaceutical and cosmetics products and for the regulation of laboratory animal experimentation. The technical challenges to developing a non-animal alternative for botulinum toxin product testing are formidable, and are best met with collaborative efforts open to scrutiny and to constructive criticism, not with alleged programs happening behind closed doors. We note for the record that none of the published studies of alternatives to LD₅₀ testing of botulinum toxin products, of which we are aware, were conducted by scientists working for the manufacturers of these products.

One of the factors that has worked against the FRAME and HSUS campaigns is the limited media interest that these efforts have generated. We suspect that this stems, in part, from limited public (and media) sympathy for mice. This is unfortunate, given that the capacity of mice to suffer is similar to that in most other animals used in laboratories. We suspect that the complexity of the relevant issues also limits the media appeal of our campaigns, including the technical nature of the non-animal alternatives and the dual use of botulinum toxin production batches for both therapeutic and cosmetic purposes. Our campaigns are also hampered by the lack of publicly available details about current testing practices and alternatives efforts.

FRAME is encouraged by the attention now being paid by the Home Office to the questions we have raised, and by the work being conducted by the NIBSC, as well as by the attention now being paid to the botulinum toxin testing issue by ECVAM and the EDQM. However, having legitimately raised an important issue of great concern in relation to both the severity of animal procedures and the need for an active commitment to finding relevant and reliable replacement alternatives, FRAME will expect progress to be made and to be kept fully informed about it.

The HSUS anticipates that its legal action against the FDA will yield critical information about the botulinum toxin testing, including the numbers of animals used per test and the number of tests conducted prior to release of a given batch of product. If Allergan continues to spurn legitimate demands for information and for co-operation, The HSUS will seek to increase the public pressure on the company, in a manner consistent with the successful campaign strategy of that late American activist, Henry Spira (Singer, 1998).

Meanwhile, both FRAME and The HSUS are willing to work collaboratively with the relevant authorities, and with institutions such as ECVAM, the EDQM, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the NIBSC, to accelerate the pace of progress, in the confident belief that we all share the same interest in making available products which are made as safe as possible for human use, but by using modern methods and progressively reducing reliance on the traditional application of painful test procedures to laboratory animals.

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