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Endotoxins in ophthalmic viscosurgical devices

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PURPOSE. *To measure the endotoxin concentration (EC) of 25 commercially available, hyaluronic acid- and hydroxypropylmethylcellulose-based (HPMC) ophthalmic viscosurgical devices (OVDs).*
METHODS. *The* in vitro *Limulus amebocyte lysate (LAL) assay, which indicates the presence of endotoxins originating from gram-negative bacteria, was used to determine the EC. The procedure was performed according to the European Pharmacopoeia/USP. EC including duplicate determinations, negative controls, dilution series with control standard endotoxin, dilution series with sample extract and positive sample control.*
RESULTS. *16 OVDs (Amvisc®, Amvisc® Plus, Biolon®, Coatel®, Healon®, Healon® GV, Healon®5, HPMC Ophtal® L, Microvisc®, Microvisc® Plus, Ocucoat®, Provisc®, Rayvisc®, Viscoat®, Visco Shield® 2%, Visko® 1.4%) had an EC under 1.2 endotoxin units/mL, five (Adatocel®, HPMC Ophtal® H, LA Gel®, Viscorneal®, Viscorneal® Plus) had an EC ≥ 1.2 and ≤ 24 EU/ml, and four (Biocorneal®, Dispasan® also named Ophthalin, Dispasan® Plus, Visko® 1%) had an EC of > 24 EU/ml.*
DISCUSSION. *To avoid viscoelastic-related inflammatory or immunological reactions, the use of pure OVDs is recommended, especially for surgical procedures with an inherent possibility of leaving viscoelastic remnants in the eye (e.g., cataract surgery, visco-canalostomy or penetrating keratoplasty). (Eur J Ophthalmol 2003; 13: 176-84)*

KEY WORDS. *Ophthalmic viscosurgical devices, Endotoxins, Limulus amebocyte lysate assay, Inflammatory reaction*

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INTRODUCTION

Ophthalmic viscosurgical devices (OVDs) have significantly helped advance surgical techniques and improve patient outcome. Various problems such as increased intraocular pressure, late secondary glau-coma, or band keratopathy, have been observed from the first OVDs to the present day (1-3). Most complications are a result of either improper injection or incomplete removal. There has been much speculation about contaminants in OVDs, and in several cases their presence has been demonstrated (4, 5). For example, after the use of Microvisc in Sweden, endophthalmitis-like reactions occurred after complication-free cataract surgeries. Tests of OVD samples by the Swedish authorities in many cases yielded an endotoxin content of up to 100 endotoxin units (EU) per milliliter. The OVD was subsequently taken off the market in Sweden (6). In Germany, too, purity problems have been occasionally reported in rinsing solutions (e.g., BSS solution by Froschek Inc.) or OVDs (e.g., Dispasan).

The aim of this study was to test the endotoxin load of 25 different recently produced sterile OVDs, ready for use in human eyes and voluntarily provided by the manufacturers. The limulus amebocyte lysate (LAL) assay is the test of choice for bacterial endotoxins because of its sensitivity, specificity, simplicity and relative lack of interfer-

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ence (7, 8). The endotoxin-mediated activation of LAL is understood and well characterized (9).

METHODS

Materials

For each of the OVDs, three units from one batch (one of two batches) were supplied, then pooled and tested for endotoxin using the LAL gel test. We employed duplicate solutions using the maximal permissible dilution of the test preparation – pretreated, if necessary, to eliminate disturbance factors. At the same time, a blank solution prepared from LAL water (see below) and two positive controls were tested, both containing endotoxin at a concentration double the stated sensitivity of the lysate. One of them contained the test preparation (pretreated, if necessary, to eliminate disturbance factors after adding an endotoxin reference substance) at the concentration used in the test. The test was only evaluated if the negative and both the positive controls gave appropriate results. The test preparation passed the test when both test solutions gave negative results. It failed if both test solutions gave positive results. If the results for one test solution were positive and the other negative, the test was repeated and the test preparation then passed if both test solutions gave negative results.

We employed a widely used LAL assay with a sensitivity of 0.06 EU/ml (Haemachem, batch number 089602). An *Escherichia coli* standard with 1000 EU (CSE, batch number: 029806) was used as reference standard. Pyrogen-free LAL water was used. The LAL water is suitable when it yields a negative result for the test preparation under the conditions prescribed for testing endotoxins. In the preparation, the water can be distilled three times in an apparatus equipped with an effective device to prevent the overflow of droplets; or it can be prepared by some other suitable process that delivers water of the required quality.

Apparatus

The following equipment was used in connection with the tests:

- sterile workbench (Holten LaminAir Safe 2000)
- pyrogen-free tools, test tubes, aluminum foil, arafilm
- water bath at a constant temperature of 37°C
- shaker
- Vortex mixer
- pH meter (WTW 540 GLP)
- ice bath for rehydrated lysate
- usual microbiological equipment.

For various test-related technical reasons (dilution of highly viscous OVDs such as Healon® 5), the limit of detection could not exceed 1.2 EU/ml.

Gel formation in a "test for bacterial endotoxins" is optimal when the pH of the mixture lies within the range of 6.0 to 7.5. The addition of lysate to the sample, however, can lower the pH. In order to make sure the pH of the mixture does not fall below 6.0, we took measures to ensure that the pH of the sample was never less than 6.5.

Test items

In response to our written request, 25 different test samples were supplied by nearly all the companies in Germany that sell OVDs (Tab. I).

Test method and preparation of the test items

The endotoxin was measured using the solid-gel method to determine the end-point. The standard limiting value of 0.5 EU/ml, on the basis of which medical devices are evaluated, and which – according to ISO/CD 15798 – will also be applied to OVDs in the future, could not be employed, for test-related technical reasons. On account of the extraordinarily high viscosity of some of the OVDs (9), the lower limit of detection, which with the lysate sensitivity is determined by OVD dilution (a minimal dilution of 1:20 was required for the processing), was 1.2 EU/ml.

With the solid-gel method, sample and lysate were mixed in a ratio of 1:1 and then incubated, completely shielded from vibrations, in a water-bath at 37° ±1°C for 60 min ±1 minute. The formation of a gel, which must remain stable when the test tube is carefully tipped, at the end of the incubation demonstrated the presence of endotoxins.

Testing for labeled lysate sensitivity (validation)

The standard endotoxin (1000 EU) was rehydrated with LAL water according to the specification and mixed until completely dissolved and homogenized for 20

TABLE I - SUMMARY DETAILS OF 25 OPHTHALMIC VISCOSURGICAL DEVICE(OVD) SAMPLES SUPPLIED FOR THE ENDOTOXIN ANALYSIS

Serial No.	Number of test samples	Type	Manufacturer/ Distributor	Batch No.	Expiration date
1	3	Ocucoat® 1 ml	Bausch & Lomb	0926	03/2000
2	3	Viscoat® 0.5 ml	Alcon	98J20	09/2001
3	3	Dispasan Plus® 15 mg/ml 0.5 ml	Ciba Vision Ophthalmics	707AA	10/2000
4	3	Dispasan® 10 mg/ml 0.5 ml	Ciba Vision Ophthalmics	712BA	03/2001
5	3	Visko® 1.4% 0.85 ml	Visional/Domilens	VC 89.14.086	05/1999
6	3	Visko® 1.0% 0.55 ml	Visional/Domilens	VC B0.10.153	07/2000
7	3	Biocorneal 1 ml	Corneal GmbH	IN 462J008	10/2000
8	3	Viscorneal 1% 0.85 ml	Corneal/Allergan	VC B1.10.142	04/2000
9	3	LA Gel Viscoelastic 1 ml	LA LABS	P1081	11/1999
10	3	Coatel® 2 ml	Chauvin Opsia	2804F	03/2001
11	3	Microvisc™ plus 14 mg/ml 0.55 ml	Bohus BioTech AB	T-032-14001	01/2000
12	3	Amvisc plus 0.8 ml	Bausch & Lomb	A9712-1	12/1999
13	3	Rayvisc™ 0.85 ml	Rayner	A 9806-5	12/1999
14	3	Microvisc™ 10 mg/ml 0.55 ml	Bohus BioTech AB	T029-10002	12/1999
15	3	Amvisc 0.8 ml	Bausch & Lomb	A9712-5B	06/1999
16	3	Adatocel 2.25 ml	Bausch & Lomb	97J27	10/2000
17	3	HPMC-Ophtal® L 1.5 ml	Dr. Winzer Pharma GmbH	621-013	05/2000
18	3	Biolon® 0.5 ml	Bio-Technology General Ltd./ Polytech	71810291	12/2000
19	3	Viscorneal + 1.4% 0.85 ml	Corneal/Allergan	VC B6.14.152	06/2000
20	3	HPMC-Ophtal® H 1 ml	Dr. Winzer Pharma GmbH	631.009	05/2000
21	3	Healon®5 23 mg/ml 0.6 ml	Pharmacia	ZF 57584	05/1999

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TABLE I - SUMMARY DETAILS OF 25 OPHTHALMIC VISCOSURGICAL DEVICE (OVD) SAMPLES SUPPLIED FOR THE ENDOTOXIN ANALYSIS

Serial No.	Number of test samples	Type	Manufacturer/ Distributor	Batch No.	Expiration date
22	3	Healon® GV 14 mg/ml 0.55 ml	Pharmacia	ZF 57566	05/2001
23	3	Visco Shield™ 2% HPMC Viscoelastic1 ml	Oasis/Domilens	V 0498C	04/2000
24	3	Healon® 10 mg/ml 0.55 ml	Pharmacia	ZI 57983	08/2001
25	3	Provisc® 0.4 ml	Alcon	98J0198J05	09/2001

TABLE II - TEST FOR LYSATE SENSITIVITY (0.06 EU/ml) AND FOR ACTIVATING OR INHIBITORY OVD PROPERTIES (validation)

Endotoxin concentration (EU/ml) result	Sensitivity	Test for activating or inhibitory OVD properties, pool of 1:200 dilutions
0.25	+/+	
0.125	+/+	+/+
0.06	+/+	+/+
0.03	+/-	+/-
0.015	-/-	-/-
LAL water	-/-	-/-

+ Gel formation - No gel formation

minutes on the Vortex mixer.

A dilution series with nine stages was prepared with LAL water: 500, 50, 1, 0.5, 0.25, 0.06, 0.03 and 0.015 EU/ml. Each test tube was mixed for approximately 30 seconds on the Vortex mixer before the next stage was prepared.

Duplicate solutions were prepared by mixing 100 µL of endotoxin dilutions 0.25, 0.125, 0.06, 0.03 and 0.015 EU/ml with 100 µL lysate, followed by incubation in a water-bath at 37°±1°C for 60min±1 minute.

Test for activating or inhibitory properties (validation)

With a pool of the 1:200 dilutions of the 25 OVDs, a dilution series was prepared as described above, and the diluted test OVD pool was used instead of the LAL water.

Endotoxin testing of the test items

The test samples were placed on a sterile pad on the safety workbench. In view of the high viscosity of the OVDs, the entire contents of the syringe were first transferred to a pyrogen-free test tube; then, depending on the contents, the same amount of distilled water was added (dilution 1:2). Since it was not possible to pipette this concentration, the dosage required to prepare the other dilutions (1:20, 1:200 and 1:400) was measured gravimetrically. The dilutions covered an endotoxin concentration range of 1.2-24 EU/ml. The maximum permissible value of 20 EU/medical device was exceeded, with a positive reaction outcome, for the 1:400 dilution.

After mixing 100 µl of the sample dilution and 100 µl lysate, all the test tubes were incubated in the water-bath at 37°±1°C for 60°±1 minute. This provided an immediate visual and manual check on whether a solid gel had formed.

RESULTS

Confirmation of lysate sensitivity (validation)

The reported lysate sensitivity (λ) of 0.06 EU/mL was confirmed (i.e. lower limit of detection). A margin of tolerance of one dilution stage either way was permissible (Tab. II).

TABLE III - ENDOTOXIN LOAD OF THE OPHTHALMIC VISCOSURGICAL DEVICES (OVDs)

Serial No.	OVD Type	Sample dilution			Endotoxin content (EU/mL)
		1:20	1:200	1:400	
1	Ocucoat®	-/-	-/+	-/-	< 1.2
2	Viscoat® 0.5 ml	-/-	-/-	-/-	< 1.2
3	Dispasan Plus® 15 mg/ml 0.5 ml	+/+	+/+	+/+	> 24
4	Dispasan® 10 mg/ml 0.5 ml	+/+	+/+	+/+	> 24
5	Visko® 1.4% 0.85 ml	-/-	-/-	-/-	< 1.2
6	Visko® 1.0% 0.55 ml	+/+	+/+	+/+	> 24
7	Biocorneal 1 ml	+/+	+/+	+/+	> 24
8	Viscorneal 1% 0.85 ml	+/+	+/-	-/-	≥ 1.2 - ≤ 24
9	LA Gel 1 ml	+/+	-/-	-/-	≥ 1.2 - ≤ 24
10	Coatel® 2 ml	-/-	-/-	-/-	< 1.2
11	Microvisc™ Plus 14 mg/ml 0.55 ml	-/-	-/-	-/-	< 1.2
12	Amvisc Plus 0.8 ml	-/-	-/-	-/-	< 1.2
13	Rayvisc™ 0.85 ml	-/-	-/-	-/-	< 1.2
14	Microvisc™ 10 mg/ml 0.55 ml	-/-	-/-	-/-	< 1.2
15	Amvisc 0.8 ml	-/-	-/-	-/-	< 1.2
16	Adatocel 2.25 ml	+/+	+/-	-/-	≥ 1.2 - ≤ 24
17	HPMC-Ophtal® L 1.5 ml	-/-	-/-	-/-	< 1.2
18	Biolon® 0.5 ml	-/-	-/-	-/-	< 1.2
19	Viscorneal + 1.4% 0.85 ml	+/+	-/-	-/-	≥ 1.2 - ≤ 24
20	HPMC-Ophtal® H 1 ml	+/+	-/-	-/-	≥ 1.2 - ≤ 24

to be continued

TABLE III - ENDOTOXIN LOAD OF THE OPHTHALMIC VISCOSURGICAL DEVICES (OVDs)

Serial No.	OVD Type	Sample dilution			Endotoxin content (EU/mL)
		1:20	1:200	1:400	
21	Healon®5 23 mg/ml 0.6 ml	-/-	-/-	-/-	< 1.2
22	Healon® GV 14 mg/ml 0.55 ml	-/-	-/-	-/-	< 1.2
23	Visco Shield™ 2% HPMC Viscoelastic 1 ml	-/-	-/-	-/-	< 1.2
24	Healon® 10 mg/ml 0.55 ml	-/-	-/-	-/-	< 1.2
25	Provisc® 0.40 ml	-/-	-/-	-/-	< 1.2

Calculation: Endotoxin concentration = Confirmed lysate sensitivity divided by the reciprocal of the sample dilution (e.g., 0.06 x 20 = 1.2 EU/ml)

Negative control:
LAL water: = Negative (- / -)

Positive control:
2 λ ≅ 0.125 EU/ml in LAL water = Positive (+ / +)
2 λ ≅ 0.125 EU/ml in the 1:200 sample dilution = Positive (+ / +)

Note: 1 λ = 0.06 EU (i.e., 2 λ = 0.125 EU with standard endotoxin)

Test for activating or inhibitory properties (validation)

The OVDs had no inhibitory effects on the test results (Tab. II).

Endotoxin load of the test samples

Of the 25 OVDs tested, 16 had endotoxin concentrations of <1.2 EU/ml, five were ≥1.2-24 EU/ml, and four had endotoxin concentrations of >24 EU/ml. The limit of detection – depending on the lysate used and the smallest possible dilution of 1:20 – was 1.2 EU/ml. Table III shows the specific results.

DISCUSSION

The use of pure OVDs is recommended in order to avoid a viscoelastic-induced inflammatory reaction, especially in operations involving a possibility of OVD residues in the eye, such as cataract surgery, viscocanalostomy or penetrating keratoplasty. In recent years, pseudoendophthalmitis-like reactions have been reported with Microvisc and Dispasan (11). In an eye clinic in Montreal, Canada, 14 cases of endophthalmitis appeared, most of them caused by bacterial contamination (*Bacillus circulans*) of the OVD (Microvisc, Q-med AB, Uppsala, Sweden, two separate batches) after a total of 42 ophthalmic surgeries (12). An en-

dotoxin content of over 100 EU/ml was discovered in 1997, while the maximum recommended content is 0.5 EU/ml (13). Florén reported that in January, 1997, in Oslo, Norway, three suspected pseudo-endophthalmitis-like reactions were observed after cataract surgery. Similar observations had also been made at other Swedish and Norwegian hospitals after using this particular batch of viscoelastic (4). This Norwegian batch, and some other batches, were contaminated by endotoxin with up to 100 EU/ml.

Pyrogen is the term used to designate substances that can trigger fever in humans in extremely small quantities. Endotoxins are lipopolysaccharides, which are components of the outer cell wall and are released upon autolytic dis-integration of bacteria. They can induce extremely violent reactions and pose a high risk of contamination of aqueous medications. Endotoxins from gram-negative microorganisms are the most frequent cause of toxic reactions, which are ascribed to contamination with pyrogens. They have much greater pyrogenic activity than most other pyrogenic substances. After intravenous injection, the symptoms range from temperature elevation to chills, leukocytosis, leukopenia, intravascular clotting, complement activation, and even shock and death, depending on the dose. In the eye, endotoxins cause an inflammatory reaction similar to uveitis. Endotoxins cannot cause bacterial endophthalmitis. The concentration at which endotoxins damage the human eye is still not known.

The only test for quantification of pyrogens in drugs that is currently recognized in the majority of countries and in the European Pharmacopoeia is the rabbit test. Rabbits and humans respond to nearly the same threshold doses of endotoxin. The reaction to the endotoxins, however, is substantially stronger in humans. According to the test in the European Pharmacopoeia, a drug batch is considered pyrogen-free when, upon injection of a certain quantity, the total of the temperature elevations in three rabbits does not exceed 1.15°C. If the term "pyrogen-free" is found on the label of a medical device or drug, then the rabbit test must have been carried out.

According to law, all manufacturers of pharmaceuticals must subject their products to a pyrogen test. This is the only such test in the German Pharmacopoeia and in the European Pharmacopoeia and accompanied by the appropriate limiting values. At present, there is no ISO limiting value for endotoxin load in medical devices like OVDs.

Endotoxin tests come in three different types: the classic, semi-quantitative test for gel formation, turbidity measurement through spectrophotometry, and the chromogenic method (14). The *in vitro* LAL test from Haemachem that was employed in this study and which is broadly applied all over the world shows the presence of endotoxin from gram-negative bacteria by gel formation (15). It is based on the reaction of endotoxins with "blood corpuscles" – the so-called amebocytes of the horse-shoe crab (*Limulus polyphemus*). The FDA and USP prescribe an endotoxin content of less than 20 EU/ampoule for medical devices. A lower limit of detection of 0.5 EU/ml (ISO/DIS 15798) is mandatory for new OVDs to be intro-duced on the inter-national market.

Beta-D-glucans, which may be found in OVDs containing hyaluronic acid as well as those with HPMC, can potentially trigger a positive reaction in the LAL test when present at certain concentrations (16). Since the hyaluronic acid preparations in this study had to be greatly diluted for testing because of their high viscosity, β-D-glucans could have influenced the reaction. We are aware that one limitation of this study is that this cannot be totally ruled out. On the other hand in the authors' opinion β-D-glucans should be extracted from any solution for intraocular use. It is well known that β-D-glucans have far less potential toxicity than endotoxins but can also induce an inflammatory reaction. Endotoxins and glucans differ in microbial origin, structure, and pharmacological action. Glucans in general are more than 1000 times less potent than lipopolysaccharides in LAL reactivity. Both agents theoretically can pass sterilizing membrane filters, so that eliminating trace contamination of either may be difficult. It has also been established that the β-D-glucan issue simply does not arise with several OVDs. So another conceivable approach is to demand β-D-glucan-free OVD from manufacturers. However, this could pose a practically insoluble problem for some manufacturers, especially those who use certain fermentative techniques to obtain hyaluronic acid. Targeted blockade of β-D-glucans to a specific extent might be called for in general before a LAL assay is conducted. Specific tests such as the owl monkey test, carried out before the release of a batch of hyaluronic acid, confirm that many viscoelastics have no in-

flammatory effects.

Although outbreaks of postoperative inflammation caused by endotoxins present in intrinsically contaminated solutions are infrequent, the very fact that they occur underscores the need for strict quality control by the producers of such solutions, strict adherence by the users of commercial OVDs to storage procedures specified by manufacturers, and heightened surveillance by ophthalmologists, hospital epidemiologists, and other control personnel for cases of postoperative inflammation associated with intraocular surgery.

CONCLUSIONS

OVDs should have low endotoxin content. Although many manufacturers claim their products are pyrogen-free, this is hardly ever attained in practice. Most of the OVDs investigated in the pre-sent study had endotoxin concentrations below the detection limit but the lowest acceptable endotoxin concentration of a substance for intraocular use has not yet been established.

Two microbial polysaccharides, characterized as lipopolysaccharide (endotoxin) and β-D-glucan, activate the LAL assay. In the present study, we were not able to determine the exact influence of β-D-glucans in differing quantities in the OVDs in which the LAL test detected endotoxins. There is no evidence that trace β-D-glucan is a real health hazard. It is im-portant to recognize that glucan-sensitive LAL tests may occasionally produce enhanced false-positive LAL-assay results.

Additional studies will further clarify the contributing factors but the ophthalmic surgeon who has not had any problems with the ophthalmic preparations in use up until now need not be unduly anxious.

Note
Using the Limulus amebocyte lysate assay, 16 ophthalmic viscosurgical devices (OVDs) had an endotoxin content under 1.2 endotoxin units/ml, 5 OVDs had 1.2-24 EU/ml, and 4 had >24 EU/ml. The use of OVDs with low endotoxin levels is recommended, especially for surgical procedures with an inherent possibility of leaving viscoelastic remnants in the eye.

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