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Bacterial endotoxin detection in hyaluronic acid-based medical devices

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Abstract: A simple and rapid method has been developed for testing bacterial endotoxin in hyaluronic acid (HA)-based medical devices. High-molecular-weight HA (HMW HA) in solution or HA-based medical devices was digested by the enzyme hyaluronidase to reduce solution viscosity by truncating the long chains of HA and to test for bacterial endotoxin. The bacterial endotoxin level was detected and measured by kinetic chromogenic *Limulus* Amebocyte Lysate (LAL) assay. The method was applied to two different ophthalmic viscosurgical devices

(OVDs) and one dermal filler, and may easily be adapted to use with up to 3% HA solutions and other HA-based medical devices. Published 2016. This article is a U.S. Government work and is in the public domain in the USA. *J Biomed Mater Res Part B: Appl Biomater*, 105B: 1210–1215, 2017.

Key Words: biomaterials, hyaluronic acid, hyaluronidase, bacterial endotoxin, medical devices, viscosity

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INTRODUCTION

Hyaluronic acid (HA) has unique biochemical and physical properties that make it an important biomaterial for several medical device applications. These include its use as ophthalmic viscoelastic device (OVD) during cataract surgery,¹ injections to treat joints with osteoarthritis², cosmetic dermal fillers³; and also its use as antiadhesion barriers in surgeries.⁴ However, once implanted, HA undergoes biodegradation which is frequently accompanied by inflammation of tissue. HA is often derived from animal tissue and bacteria, and may be contaminated with bacterial endotoxin.⁵ Bacterial endotoxin (lipopolysaccharides (LPS) from outer membrane of Gram-negative bacteria) can cause painful inflammation, leading to failure of the device. The U.S. Food and Drug Administration (FDA) recommends a maximum allowed limit of endotoxin that may be present in medical devices which are in contact with blood, cerebrospinal fluid, or implanted in the body⁵; or single-use intraocular ophthalmic devices.⁶ Bacterial endotoxin contamination may be introduced at any stage of the HA-based medical device manufacturing process. There are several potential sources of the endotoxin contamination: water, chemicals, and raw materials involved in manufacturing, packaging components, equipment, and factory employees. The quality and purity of all materials involved in manufacturing have to be established and controlled at every stage of the process. A final medical device product, that is intended for implantation in general or intended to come in contact with

blood or cerebrospinal fluid, or ocular tissues, has to meet its endotoxin limit by being tested for bacterial endotoxin.^{5,6}

Several reports suggested that HA-based products may have been contaminated with bacterial endotoxin⁵; and in effect, may have caused inflammatory reactions in patients who received short- or long-term implantation.^{2,7,8} OVDs fabricated from HMW HA are indicated for use during cataract surgery.⁹ Toxic anterior segment syndrome (TASS) is a noninfectious eye inflammation that can occur after cataract surgery, leading to glaucoma and damaging the cornea and retina.¹⁰ It has been demonstrated that OVDs composed of high molecular weight HA, and contaminated with bacterial endotoxin, may cause TASS.¹¹ A number of TASS outbreaks over the past 11 years have affected patients from many surgical centers in North America.¹² FDA researchers investigated the causes of TASS outbreaks as adverse events¹³; and developed new testing methods to determine the inflammatory potential of suspected contamination of subject devices.^{14–21}

In 2012, Buchen et al. evaluated an inflammatory potential of cohesive and dispersive OVDs which were spiked with known endotoxin concentrations,¹³ and were measured using the LAL kinetic turbidimetric assay. Rabbits were given known concentrations of bacterial endotoxins mixed with HA and the researchers noted the concentration at which of endotoxin caused inflammation in the eye. They also noted that because the HA was so viscous, it was difficult to obtain accurate amount of samples to measure

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bacterial endotoxins using the LAL assay.¹³ Dermal fillers require minimal surgical intercession because they can be easily delivered under the skin via injection. This fact led to the development and approval of several HA-based dermal fillers since the first one cleared by FDA in 2003. According to FDA's Medical Device Reports (MDR), however, there were 930 adverse events associated with use of the dermal fillers between 2003 and September 20, 2008.¹⁶ These events may have occurred due to the surgical procedure technique (depth and the correct placement of injection itself), concentration of the product used (excessive amount), protein and endotoxin contamination, and low-molecular-weight HA fragments generated during biological degradation.^{8,17–20}

The focus of this study is to develop a simple and rapid method for testing the presence of bacterial endotoxin in HA-based medical devices. High-molecular-weight HA samples were first digested using hyaluronidase enzyme and then, the *Limulus* amoebocyte lysate (LAL) chromogenic kinetic test method was used to detect and quantify the amount of bacterial endotoxin in the HA digests. Out of three different commercially available sources of hyaluronidases, we found two were contaminated with bacterial endotoxin. We therefore used the clinically available hyaluronidase (i.e., recombinant hyaluronidase), which had no endotoxin contamination, to digest three different HA-based medical devices.

We found that hyaluronidase digestion of high viscosity HA prior to endotoxin measurements can greatly aid the detection of bacterial endotoxins in implantable HA-based medical devices.

MATERIALS AND METHODS

Hyaluronic acid

Dry HA powder samples with the average molecular weights of 1.2–1.8 MDa and low endotoxin content (specification at less than 0.07 EU/mg) were purchased from LifeCore Biomedical, LLC (Chaska, MN, USA). The research grade sodium hyaluronate was derived from pharmaceutical grade batches and met the Lifecore Biomedical's sodium hyaluronate pharmaceutical specifications. The HA samples were solubilized in sterile endotoxin-free water (Lonza) at 2.0 and also, at 20.0 mg/mL.

HA-based medical devices

Samples of commercially available OVDs, containing 1% and 3% HA were tested. Upon the manufacturer's recommendations, they were stored at 2°–8°C before use. The dermal filler was a gel of HA produced by *Streptococcus* species, chemically cross-linked with BDDE (1, 4-butane-diol diglycidyl ether), stabilized and suspended in phosphate buffered saline at pH = 7 and at HA concentration of 20 mg/mL. The dermal filler was stored at up to 25°C with no exposure to direct sunlight upon the manufacturer's recommendations.

Hyaluronidase

Hyaluronidases from bovine testes and from *Streptomyces hyalurolyticus* were purchased from Sigma-Aldrich Chemical

Co. (St Louis, MO, USA). Following the manufacturer's instructions, the testicular enzyme was dissolved in cold 20 mM sodium phosphate buffer and used at 1,450 U/mL. Also following the same, the *Streptomyces* was dissolved in pH 7.0 with 77 mM sodium chloride or in endotoxin-free water, and used at 200 U/mL. The recombinant hyaluronidase solution (150 USP units/mL) was purchased from Halozyme Therapeutics, Inc. (San Diego, CA, USA) and following the manufacturer's recommendations, it was stored in a refrigerator at 2°–8°C. Each milliliter of the recombinant hyaluronidase solution contained 150 USP units of recombinant human hyaluronidase with 8.5 mg sodium chloride, 1.4 mg dibasic sodium phosphate, 1 mg albumin human, 0.9 mg EDTA, 0.3 mg calcium chloride, and sodium hydroxide added for pH adjustment. The enzyme solution pH was 7.0.

Lipopolysaccharide (endotoxin)

LPS from *Escherichia coli* serotype O26:B6 (endotoxin), purchased from Sigma Aldrich, was reconstituted with dimethyl sulfoxide (DMSO, Sigma) to a concentration of 250 µg/mL and frozen in aliquots at –70°C until use.

Enzymatic digestion

The HA samples and HA-based medical devices were digested by adding 40 units of recombinant hyaluronidase to 1 mL of HA sample and mixing for 10 s at high speed with a vortex mixer. All digestion experiments were performed at room temperature, and the samples were shaken on a 55D Double Platform Shaker for 24 h at 55 rpm at room temperature. Sample degradation was confirmed by observing changes of viscosity of the samples. The digested HA samples were stored in a freezer at –20°C prior to LAL assay.

Endotoxin detection

Endotoxin was assayed according to the instructions with the Lonza Kinetic-QCL Chromogenic *Limulus* Amoebocyte Lysate (LAL) Endotoxin Assay kit with a sensitivity range of 0.005–50.0 EU/mL (Walkersville, MD). Both positive and negative controls were included with each assay. A set of standards of known concentrations of bacterial endotoxins was included on each assay run. The correlation coefficients, or R^2 values, for the standard curves were between 0.9855 and 0.9957.

Viscosity measurements

Using a rotational rheometer (AR-G2, TA instruments, Delaware, USA) with a 60-mm-diameter stainless-steel cone-and-plate geometry (truncation angle 2°), viscosity was measured at 0.1/s shear rate for 30 min at room temperature before enzyme addition. Enzyme was added to HA on the rheometer plate and the viscosity was measured again, at the same rate for 30 min immediately after enzyme addition and from 1.25 to 1.75 h after enzyme addition. Using the same experimental conditions, shear viscosities of HA chains were also measured between 0.01/s and 10/s shear rates, followed by an immediate reverse cycle from 10/s to 0.01/s shear rates. High-concentration (3%) HA sample from OVD

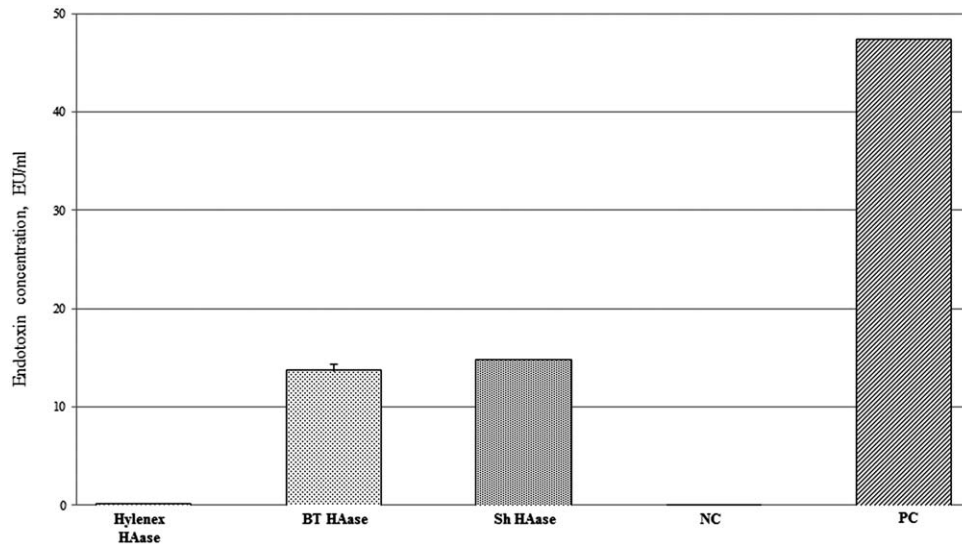


FIGURE 1. Average of endotoxin levels in three different sources hyaluronidases: recombinant (Hylenex HAase), Bovine testes (BT HAase), *Streptomyces hyalurolyticus* (Sh HAase), and in the positive and negative controls (PC and NC), respectively. The endotoxin activity of the hyaluronidases was determined by kinetic chromogenic LAL assay. Values in bar graphs shown represent the means of three experiments \pm standard deviation (SD) of 3 replicate wells per sample.

was used to confirm reduction in viscosity by enzymatic degradation; and rooster comb HA from Sigma–Aldrich was prepared at 3% concentration and tested using the same protocol for comparing changes in viscosity.

RESULTS

In this study, we developed a simple and rapid method for testing for the presence of bacterial endotoxin in high-molecular-weight HA-based medical devices. Enzymatic digestion of HA prior to endotoxin measurements is a method which exposes the HA bacterial endotoxin, thereby allowing it to be measured easily using the LAL assay.

Hyaluronidases testing for the bacterial endotoxin

Figure 1 represents that the bacterial endotoxins in hyaluronidases from bovine testes (BT HAase) were at 13.18 EU/mL and that in *Streptomyces hyalurolyticus* (Sh HAase) were at 14.7 EU/mL. Both the recombinant human hyaluronidase and the endotoxin-free water tested negative for the presence of endotoxin. The recombinant human HAase was chosen for enzymatic degradation of all our HA test samples.

Bacterial endotoxin detection in HA solutions and HA-based medical devices

Figure 2 shows the results of the bacterial endotoxin detected in enzyme-digested HA samples. The manufacturer

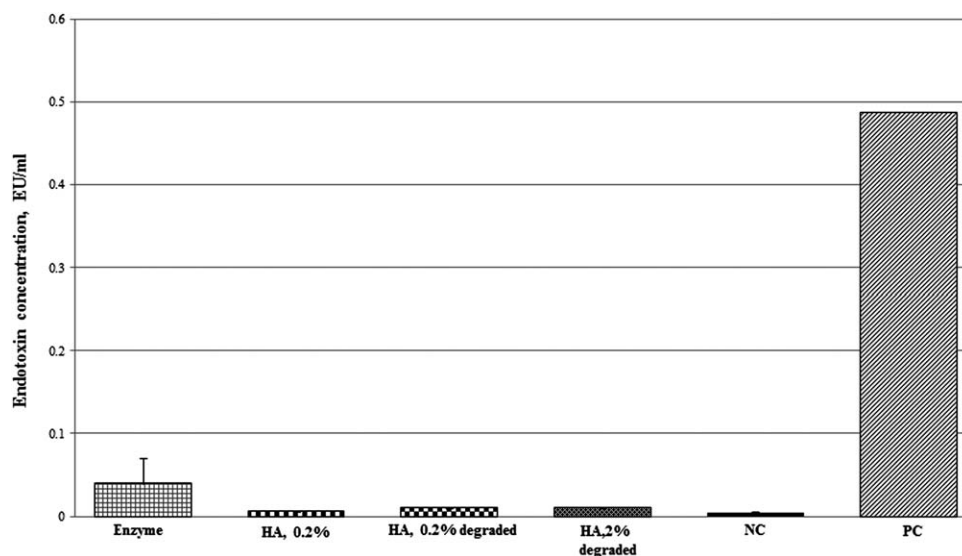


FIGURE 2. Endotoxin detection in hyaluronic acid of two concentrations, 0.2% and 2.0% before and after degradation for 24 h and hyaluronidase at 150 U/mL (enzyme), and in the positive and negative controls (PC and NC), respectively. The endotoxin activity of the samples was determined by kinetic-chromogenic LAL assay. Values in bar graphs shown represent the means of three experiments \pm SD of 3 replicate wells per sample.

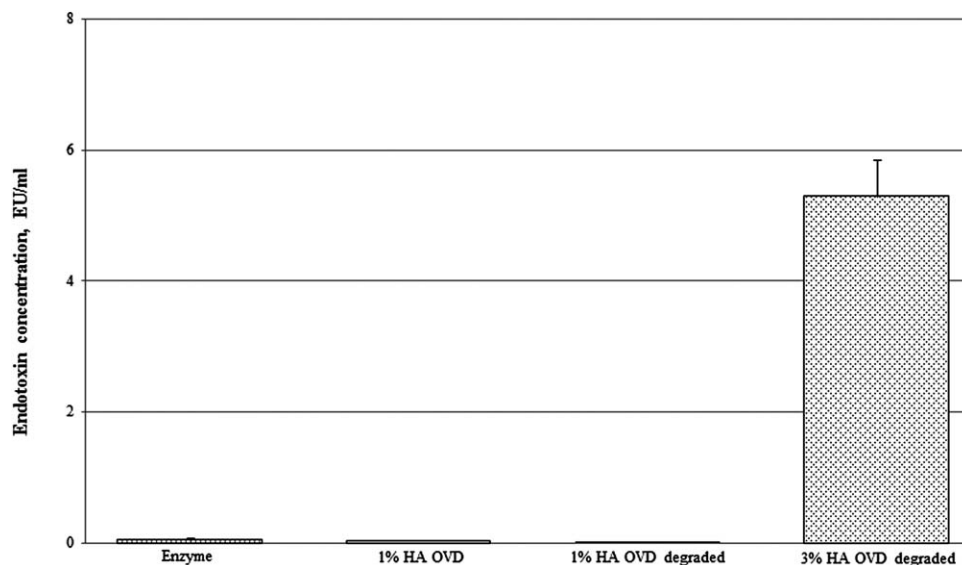


FIGURE 3. The average of endotoxin levels in 1% HA OVD and 3% HA OVD (HA-based ophthalmic viscosurgical devices at 1.0% and 3% of HA, respectively) before and after digestion for 24 h and hyaluronidase at 150 U/mL (enzyme). The endotoxin activity of the samples was determined by kinetic chromogenic LAL assay. Values in bar graphs shown represent the means of three experiments \pm SD of 3 replicate wells per sample.

of these samples claimed <0.07 EU/mg of endotoxin when diluted in sterile endotoxin-free water with low endotoxin content. The HA samples were used at two different concentrations, 0.2% and 2.0%; and after an enzymatic digestion, neither of the HA samples showed any detectable level of the bacterial endotoxin when tested for and compared to the negative control (NC, endotoxin-free water) and positive control (PC, *E. coli* 055:B5 Endotoxin standard at 0.5 EU/mL).

Figure 3 represents the bacterial endotoxin detection in enzyme-digested original solutions (1.0% and 3.0%) of OVDs compared to two of the assay controls: negative, NP (endotoxin-free water) and positive, PC (LAL assay standard at 50 EU/mL). Three independent experiments were performed with each sample and three replicas per test condition. Results suggest that the 3% HA OVD was contaminated with the bacterial endotoxin from the outset, whereas the 1% HA OVD showed no detectable level of the bacterial endotoxin. Nondigested 3% HA OVD alone cannot be assayed for the presence of bacterial endotoxin because of its high viscosity¹³ and difficulty in handling small volumes accurately.

Figure 4 shows the results of bacterial endotoxin detection in 2% HA-based dermal filler, as received or spiked with 24 EU/mL of bacterial endotoxin, which were then digested prior to testing. Two independent experiments were performed with each set of samples with either four or six replicas per test condition. The results for nondigested and digested dermal fillers, which were not spiked with LPS, showed absence of significant amounts of endotoxin when assayed. However, the digested endotoxin-spiked dermal filler revealed higher bacterial endotoxin level than the nondigested sample.

Viscosity

Figure 5 confirms the change in viscosity of 3% OVD sample after enzymatic degradation, and compares it with the same

for 3% rooster comb HA samples. At a constant shear rate of 0.1/s, the viscosity of both samples are constants and comparable for the first half hour. The viscosity of HA from rooster comb (RC) declined at much faster rate than that from OVD, immediately within the first half hour after the enzyme addition. When measured again between 1.25 and 1.75 h after enzyme addition, the viscosities values of both samples reduced considerably from their initial values. In addition, shear viscosities of each sample, which were comparable for both the forward (–) and the reverse (–) curves, changed considerably immediately after enzyme addition (middle and right panel in Figure 5). Noticeably, the viscosity on RC samples declined to ~ 2 Pa s within the first hour, while that of the OVD sample changed to 4–5 Pa s after 8 h. However, it is a 10-fold decrease from its original value and in the actual experiment, the samples were treated for 24 h with enzyme, and before running LAL assay.

DISCUSSION

Reagents or chemicals that are used in research and in manufacturing of medical devices can be contaminated. Possible sources of contamination include water, raw materials that contain lipopeptides, peptidoglycans, endotoxin, or other proteins.^{14,22} Bacterial endotoxin is known to bind to other chemicals as well as to many surfaces,²³ revealing lower than tangible levels in the actual device. For example, the LAL assay could be exaggerated (3% HA of OVD) or underestimated (endotoxin-spiked dermal filler), especially in high-viscosity HA solutions or gels.

Our results suggest that in HA, bacterial endotoxin detected by LAL kinetic assay was at least 2 times lower than in an endotoxin solution at 24 EU/mL alone. Since the molecular weight of a polymer chain is proportional to its viscosity, and OVDs (containing 3% HA) and dermal fillers

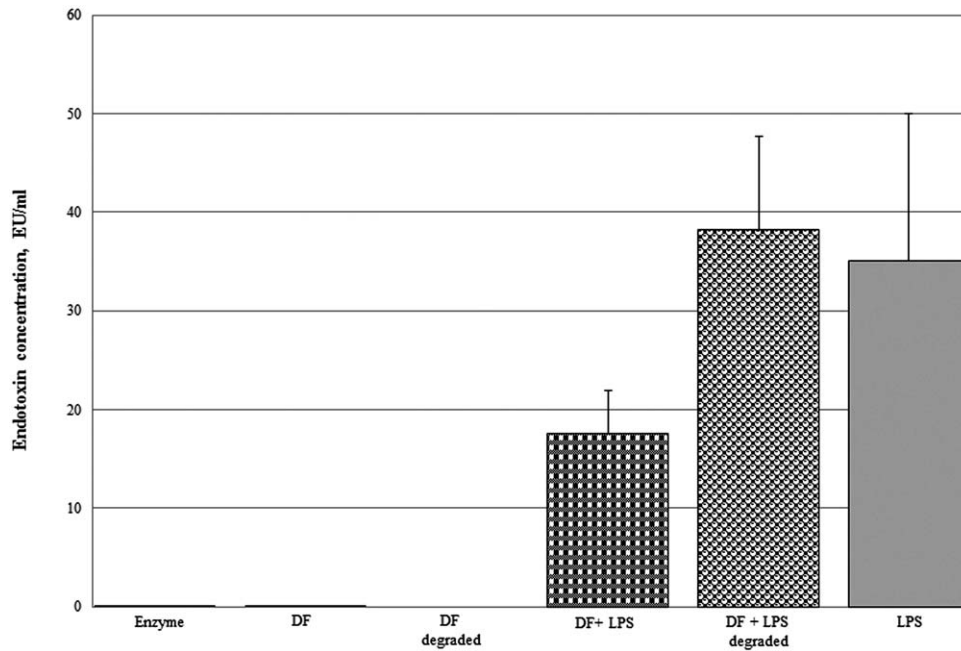


FIGURE 4. Average of endotoxin levels in HA-based dermal filler (DF) and HA-based dermal filler spiked with endotoxin at 24 EU/mL (DF + LPS) before and after digestion for 24 h and in hyaluronidase at 150 U/mL (Enzyme), and endotoxin at 24 EU/mL (LPS). The endotoxin activity of the samples was determined by kinetic chromogenic LAL assay. Values in bar graphs shown represent the means of two experiments \pm SD of 4 replicate wells per sample.

(containing 2% HA) are high viscosity solutions (Figure 5), they contain long-chain HA molecules.

Figure 5 shows that under a steady flow, comparable concentrations of HA from two different sources (OVD and rooster comb) have high viscosities toward the “zero shear” limit, but fairly low viscosity at higher shear rates which

recovered to the original viscosity value during reverse tracking (Figure 5, middle and right panels). This recovery suggested the presence of entangled HA chains which opened up under high shear; otherwise if the decrease in viscosity at a high shear was due to chain scission, it would not have recovered close to the original value. Viscosity

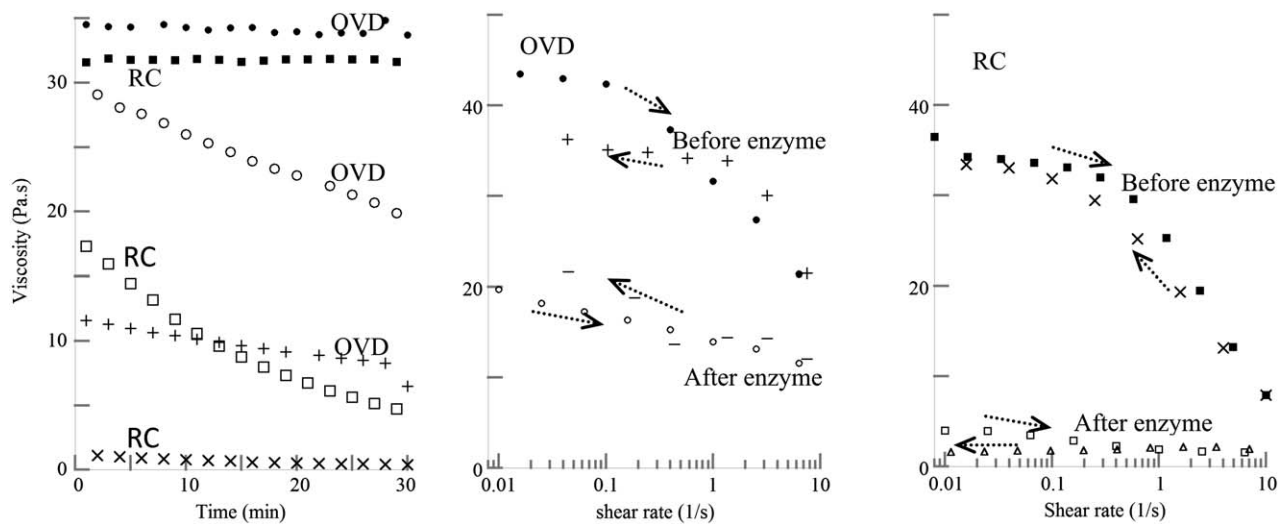


FIGURE 5. Comparison of viscosities of 3% ophthalmic HA (OVD) sample (\bullet , \circ , $+$) and 3% rooster comb HA (RC) (\blacksquare , \square , \times) samples; and shows that the viscosity values for both the original samples are in close range, the rate of change in viscosities after enzyme addition varied tremendously; however, the trends are similar. *Left panel* represents the respective viscosities of OVD and RC samples at a constant 0.1/s shear rate over time prior to enzyme addition (\bullet , \blacksquare) and up to half after (\circ , \square) after enzyme addition, and also, within 1.25–1.75 h ($+$, \times) since enzyme addition. *Middle panel* represents the shear viscosities of the OVD samples with increasing shear rate (\bullet) followed by an immediate decreasing shear rate ($+$) before enzyme addition, and the same (\circ , $-$) respectively) immediately after enzyme addition. *Right panel* represents the shear viscosities of RC samples with increasing (\blacksquare) and immediately decreasing shear rate (\times) before enzyme addition, and the respective (\square , Δ) immediately after enzyme addition.

measurements confirmed that enzyme application reduced the viscosity of the highest concentration HA solution significantly, which is a signature of reduction in molecular weight and therefore a reduction in entanglement. Such entanglements in the original HA-solutions may have concealed some of the bacterial endotoxin within the chain folds. Therefore, it may be necessary to use endotoxin-free hyaluronidase to break down the large HA molecule and make endotoxin more accessible for LAL testing, which is otherwise performed under immobile conditions, that is, in the presence of entanglements. In this study, a simple method was developed to show that digestion of HA by hyaluronidase prior to LAL assay can be used to accurately account for bacterial endotoxin in highly viscous HA solutions can be obtained after its digestion. This HAase treated HA method may allow detection of the presence of bacterial endotoxin without the use of animals.²⁰ The results of this study may help to increase the accuracy of endotoxin measurements by LAL assay, to ensure safety of the HA-based medical devices and help to evaluate for this potential cause of inflammation by these devices. Detection of bacterial endotoxin during the manufacturing processes can be monitored to allow the manufacturers to detect and measure the presence of bacterial endotoxin in their final products.

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