Perspectives on the Use of Hyaluronidase from the Red King Crab Hepatopancreas in Treating Filler Complications: In Vitro Investigations

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1. Introduction

The increasing popularity and relatively affordable prices of contour plastic using HA-based fillers are followed by an increasing rate of cases of unwelcome effects and hypercorrection [1]. The main complaints of patients after contour plastic are dissatisfaction with the aesthetic appearance, swelling, and hypercorrection. Sometimes, the HA injection triggers the process of acute inflammation, which requires quick intervention and removal of HA. Development of safe and effective way to rule out the negative effects of HA injections is therefore an urgent issue for cosmetology and medicine. Moreover, it is known that a high content of HA around cancer cells increases their resistance to the immune system, positively influences the ability of cancer cells to migrate and therefore raises probability of metastatic spreading [2]. As a consequence, the use of hyaluronidases in the treatment of cancer is crucial issue.

HA is a high molecular weight negatively charged polymer, glycosaminoglycan, consisting of repeating disaccharide units (D-glucuronic acid and N-acetyl-D-glucosamine) linked by β-1,4 and β-1,3 glycosidic bonds. Depending on the source of HA, disaccharide units can be repeated 2000–25000 times (10^6–10^7 Da). In an aqueous environment, HA has a complex secondary and tertiary structure [3]. In an electron microscope, HA is observed as a cluster of interwoven filaments or star-shaped leaves formations, the filaments can intertwine [4,5]. It forms loops and loose glomus. Quite the same result was obtained by AFM method [6]. HA is involved in the proliferation, differentiation and migration of cells, also it is the main component of the synovial fluid and extracellular matrix, and it regulates water balance [7].
Due to the chemical properties of HA, numerous approaches were developed for its chemical modification, on an industrial scale as well, which contributed to the emergence of a number of HA forms of medications successfully used in medicine and cosmetology [8]. Most commercially available fillers contain high molecular weight (about 1 MDa or even higher) and low molecular weight (several hundred kDa) HA molecules. Furthermore, high-molecular weight HA molecules often contain transverse covalent cross-links between themselves, and therefore they are less susceptible to degradation by endogenous hyaluronidases [9]. Low molecular weight forms of HA do not contain cross-linking and provide lubrication during injection. The ratio of the two forms varies from product to product (Table A1).

Up to the present the hyaluronidase injection directly into the HA area is the most reliable and painless way to manage the adverse events. A positive effect is achieved because the enzyme degrades the polymeric form of HA to its monomers. HA-based fillers produced by different manufacturers are degraded in different efficiency by hyaluronidase. In vitro studies have shown differences in degradation of cosmetic HA preparations with hyaluronidase (bovine or ovine testis). Thus, determination of the correct dosage of hyaluronidase is quite a challenge [9–11].

Previously hyaluronidase activity was detected in the enzyme complex of the hepatopancreas homogenate of the red king crab Paralithodes camtschaticus [12]. Immediately after incubation with the hepatopancreas collag enolytic protease complex the HA molecular weight was estimated with the help of capillary viscometer method. The authors tend to think that the hyaluronidase activity belongs to some (or several) of the nine proteases of that complex. Indeed, enzymes broad specificity were described in several studies. For example, some lipase and papain enzymes hydrolyze chitosan [13,14]. It is also likely that hepatopancreas of the red king crab in addition to the proteases, contains hyaluronidase. Representatives of malacostraca class (lobster Nephrops norvegicus, krill Euphausia superba), which includes the red king crab as well, contain hyaluronidases in their hepatopancreas. Their biochemical properties have been characterized [15,16]. The hepatopancreas hyaluronidase activity of some shrimps has been shown as well [17]. At the moment, the structure of hyaluronidases of these animals has not been studied at all. In the GenBank database there is only one predicted gene of a representative of this order. It is a shrimp Penaeus vannamei, NCBI Reference Sequence: XM_027376109.1.

Summing up, the commercial species of crustaceans are a promising source of hyaluronidase production for the needs of cosmetology and medicine. Considering that hepatopancreas of the red king crab is a commercial waste, this raw material is available for large-scale processing in order to obtain a cosmetic hyaluronidase product.

In this study, using the turbidimetric method and AFM, there has been investigated the fillers HA degradation with the help of commercially available hyaluronidases and the hyaluronidase of the red king crab hepatopancreas. The results of this work show the comparable effectiveness of the studied hyaluronidases and consequently demonstrate the opportunities to create a new hyaluronidase product based on the red king crab hepatopancreas. In addition, the results of AFM and NMR provide insights into the structural transformations of filler HA following exposure of the hyaluronidase of the red king crab hepatopancreas.

2. Results

2.1. Turbidimetric analysis of HA-based fillers

Turbidimetric method was used for determination of hyaluronidase activity. The turbidimetric method, in contrast to the method for measuring the viscosity of HA solution, is less laborious and requires a small amount of substrate. This method is based both on the property of high molecular weight HA to form bonds with albumin in acidic conditions and form aggregates. Scattered light of the aggregates is registered by the light-scattering method or the optical density measurement of the sample. As high molecular weight forms of HA transit to low molecular weight (due to hydrolysis), the
ability to form aggregates disappears. This phenomenon underlies the determination of hyaluronidase activity by this method and allows kinetic studies. Previously, it was implicitly shown that the average molecular weight of HA, at which it stops to form aggregates with acidic albumin, is 6–8 kDA [18].

Figure 1(a) demonstrates the concentration dependence of optical density on amount of HA registered by turbidimetric analysis. The curves vary for different HA-based filler manufacturers, that is probably connected to the difference in the ratio of cross-linked to free form of HA and in molecular masses of HA in the fillers, which are likely to bind to acidic albumin with different efficiencies. In further studies, the optical density values were considered as correct if the values were on a linear part of these curves. It is important to note that unmodified HA obtained from rooster comb binds to albumin much more effective.

Figure 1. The study of HA of rooster comb, Revofil Ultra, Hyaluform and Teosyal Ultra fillers HA in turbidimetric analysis: (a)—concentration dependence of the amount of HA of rooster comb, Revofil Ultra, Hyaluform and Teosyal Ultra and optical density in turbidimetric analysis, (b)—kinetics of hydrolysis of Revofil Ultra filler HA using HPC homogenate in triplicate, (c)—kinetics of the hydrolysis of the Hyaluform filler HA using commercially available hyaluronidases and HPC homogenate, (d)—kinetics of hydrolysis of Revofil Ultra filler HA using three different concentrations of HPC homogenate protein, (e)—kinetics of hydrolysis of Revofil Ultra filler HA using HPC homogenate before and after increasing the temperature of the reaction mixture to 100 °C for 10 minutes, (f)—kinetics of hydrolysis of the Hyaluform filler HA using the HPC homogenate and its decontaminated sample («decont. HPC»).

Turbidimetric analysis of filler HA hydrolysates induced by the homogenate of the red king crab hepatopancreas (HPC) showed an unexpected result (Figure 1(b)). Instead of the anticipated reduced HA capacity to form aggregates with acidic albumin ([19] and Figure A1), the opposite effect was observed in the first 40 minutes—the reaction products of hydrolysis of the filler HA bound the protein and formed aggregates with it more effectively (optical density increases). After 40 minutes of the reaction time, the optical density in the turbidimetric samples decreased. The same result of HA hydrolysis of the Hyaluform (Figure 1(e)) and Revofil Ultra (Figure A2) was obtained for commercially available hyaluronidases Lydase and Liporase.
The dependence of the hydrolysis efficiency on the amount of total HPC homogenate protein in the reaction mixture has been investigated in the present study (Figure 1(d)). The increase of concentration resulted in hydrolysis of HA into fragments that are incapable to bind to acidic albumin at a quick rate. With a ratio 1 to 1 (by weight) of the total HPC homogenate protein to HA, on the 10th minute of hydrolysis an insignificant level of aggregation was detected, that is, within 10 minutes almost all HA turned into short forms. When concentration of the enzyme in the reaction mixture decreased, HA transited to such short forms more slowly. The hydrolysis time, at which the more effective HA forms in terms of binding to albumin formed, shifted to later time points: in the ratio 1:5 (protein to HA) peak on the 10th minute, in the ratio 1:10 peak on the 20th minute. The ratio of 1:10 or 1:15 was taken as optimal for the experiments.

Raising the temperature of the reaction mixture to 100 °C for 10 minutes allowed to stop the hydrolysis reaction: after 40 minutes of incubation at 37 °C the reaction mixture (in tightly closed eppendorf tube) was placed for 10 minutes in a boiling water bath, after which the incubation continued at room temperature (Figure 1(e)). Such a simple approach will make it easy to obtain partial hydrolysated cross-linked HA and to study them in future researches (see Discussion).

2.2. Atomic force microscopy of HA-based fillers

To study HA hydrolysates using AFM, the sample of the HPC homogenate had to be decontaminated of pigments and other non-protein contaminants (see Materials and Methods). Decontaminated homogenate showed the same level of hyaluronidase activity as the initial one (Figure 1(ff)). SDS-PAGE protein electrophoresis did not reveal significant differences in the set of proteins between these two samples of the preparation (Figure A3).

The AFM images of the HPC homogenate 21 times diluted by phosphate buffer showed the presence of amorphous structures, but the decontaminated homogenate resembled «globular»-like structures with 4 to 30 nm in height (Figure A4). Probably, these are complexes of proteolytic and other enzymes.

As filler HA solutions were stored at 4 °C, the samples were preliminary warmed up at 37 °C for 40 min to be studied using AFM. The heat treatment did not significantly affect the results of turbidimetric analysis (Figure A5). The AFM images of Hyaluform filler showed spherical-like structures from several nm to 300 nm in height (Figure 2).

![AFM images of Hyaluform HA-based filler](image)

**Figure 2.** The AFM images of Hyaluform HA-based filler, obtained in the tapping mode: (a)—field 50x50 µm, (b)—field 1.4x1.4 µm.

Population of these spherical-like structures can be divided into 3 groups by size and quantity. The first group includes small-numbered population structures 150–300 nm in height (Figure 2(a)). The similar structures were observed in Revofil Ultra filler preparation but the structures were up to 150 nm in height (Figure A6). The second group is represented by large population structures 10–20 nm in height. The much numerous structures several nm in height were attributed to the third group. These group forms a dense layer surrounding structures mentioned above (Figure 2(b)).

AFM method was used to study the hydrolyzates of Hyaluform filler HA. Hydrolyzates were obtained with the decontaminated HPC homogenate. The aliquots were picked out from the reaction
mixture at the 5, 40 and 120 min of incubation of the reaction mixture and were transferred to mica to be studied using AFM (Figure 3, Figure A7).

![AFM images](image1)

**Figure 3.** AFM images in the tapping mode of the hydrolyzates of Hyaluform HA-based filler: a—5 min, b—40 min, c—120 min of the hydrolysis of HA with the HPC homogenate.

It was found that at time point of 5 min of hydrolysis the empty spaces without presence of HA are formed in the layer of spherical-like structures (Figure 3(a)). After 40 min of the hydrolysis these empty spaces expanded (Figure 3(b)). After 120 min of the hydrolysis the spherical-like structures almost disappeared and a filamentous mesh structures formed (Figure 3(c)). The filamentous mesh structures are similar to those resulted from AFM imaging of noncross-linked HA presented in the paper of Jacoboni et al [20].

Besides, the AFM image of the reaction mixture after 5 min of incubation showed also the spherical-like structures about 600 nm in height and greater (Figure A8). After 40 min of the hydrolysis the number of these big structures decreased and there were many spherical-like structures up to 150 nm in height. After 120 min of incubation the structures up to 80 nm were seen rarely, sometimes the amorphous structures were visible.

### 2.3. Nuclear magnetic resonance of HA-based filler

NMR spectrum of hyaluform HA has revealed a weak signal of $^1$H of sugar rings in a range of 5.9–3.3 ppm and a minor signal of $^1$H of acetyl groups of N-acetyl-D-glucosamine in a range of 2 ppm (Figure 4). The detected low level of signals is due to close package of high molecular weight cross-linked molecules of filler HA.

The reaction mixture of hydrolysis of the Hyaluform filler by purified HPA homogenate was incubated in NMR spectrometer at 37 °C for several days and NMR analysis was performed. Figure 4 shows a scan of the 5th day of incubation of the sample. It is seen that protons ($^1$H) of NH group, sugar rings and acetyl groups of N-acetyl-D-glucosamine have significantly higher signals in comparison with initial HA. No significant proton signals of acetic acid, amino acids or other compounds were detected in the spectrum, which could indicate contamination of the sample with bacterial population during the incubation. In Figure 5(a) changes in the spectra of protons of acetyl groups of N-acetyl-D-glucosamine during the experiment are represented in details. The protons of the acetyl group of HA in a free state give a symmetric narrow signal in the NMR spectrum, while the protons of the same acetyl group of HA, but included into the associates, give a wide asymmetric signal which is shifted to a high field by 0.04 ppm. The asymmetry of the peak is due to different sizes of HA associates.
in solution. Based on the results the accumulation kinetics of signal from $^1$H of free acetyl groups of N-acetyl-D-glucosamine (Figure 5(b)) was obtained under experimental conditions corresponding to the conditions in turbidimetric analysis (Figure 1(f)). According to NMR analysis HA degradation in the reaction mixture lasts for several days, but the maximum reaction rate is observed in the first hours of incubation.

**Figure 4.** $^1$H-NMR spectra of Hyaluform filler and its hydrolysis products after treatment by purified HPC homogenate. Black curve—Hyaluform filler, red curve—hydrolysis products after 5 days of incubation.

**Figure 5.** Hydrolysis of Hyaluform HA by purified HPC homogenate. (a)—changes in signals of $^1$H of acetyl groups of N–acetyl–D–glucosamine in $^1$H–NMR spectra; (b)—accumulation kinetics of signal from $^1$H of free acetyl groups of N–acetyl–D–glucosamine (integral under the peaks).
3. Discussion

This study focused on hydrolysis of cosmetic fillers hyaluronic acid (HA) and kinetics of the HA hydrolysis using the homogenate of the red king crab hepatopancreas. The turbidimetric analysis showed the bell-shaped dependence between the efficiency to form aggregates with albumin and reaction time. The ascending part of the turbidimetric curve could be explained by accumulation of products of HA hydrolysis in reaction mixture which are more effective to form aggregates with albumin than initial one. The descending part of the curve is degradation of the HA molecules to short forms which are unable to form the aggregates with albumin.

Using AFM it was shown that the HA of the fillers has a spherical-like structure in the size range from several nm to several hundreds nm. Obviously, such a structural organization should be more stable than non-modified HA to the action of hyaluronidase, since only the surface molecules of HA of the structures can undergo hydrolysis, while the molecules inside are protected from the action of enzymes. Under the action of the hyaluronidase of the HPC homogenate, these structures decompose into a filamentous mesh structure.

The observed big spherical structures with a height of approximately 600 nm (Figure A8) can be explained by the attaching of proteins of the enzyme preparation into HA in the reaction mixture. Earlier, Flynn et al. investigated the kinetics of changes in the molecular weight of HA of the Belotero Balance, Restylane, and Juvéderm Ultra fillers during a hydrolysis reaction using ovine testicular hyaluronidase [10]. The results of liquid chromatography showed an increase in the molecular weight of HA in the first 30 minutes of the reaction. The authors explained this phenomenon as the result of the formation of enzyme-substrate complexes during the reaction. Most likely, the enlarged spherical-like structures observed by us in the first 5–40 minutes of the reaction and the detected increase in the molecular weight of HA in the work of Flynn et al. have the same basis.

The accumulation of signal from \(^1\text{H}\) of free acetyl groups of N-acetyl-D-glucosamine during the incubation of Hyaluform filler with purified HPC homogenate indicates the kinetics of enzyme activity. As seen, the hydrolysis lasts for several days while the turbidimetric analysis curve reaches plateau in three hours under the same experimental conditions. This is obviously due to a limitation turbidimetric assay method wherein the formation of aggregates is very sensitive to the size of hyaluronic acid.

The HPC homogenate has hyaluronidase activity comparable to commercially available products, if the total protein concentration of the compared hyaluronidase preparations is equalized in the reaction mixtures. Thus, for the first time in this work, we have demonstrated the prospects of using hepatopancreas of the red king crab as resource to create a new hyaluronidase product for treating filler complications.

Products obtained from mammalian tissues contain a potential danger to the consumer if the animal has spongiform encephalopathy. Prion proteins of such an animal can cause development of Creutzfeldt-Jakob disease in humans. Despite the fact that at the moment no case of this route of infection through the hyaluronidase preparations has been described, state regulators of many countries prohibit the import and sale of these products on their territory. An exception is hyaluronidase preparations obtained from tissues of the testes of bulls grown in Australia. A quarantine regime of animal importation has been introduced in this territory, and it is believed that
the local cattle population has never been exposed to spongiform encephalopathy. In this regard, the preparation of the red king crab hepatopancreas hyaluronidase has a significant advantage, since it cannot carry such a potential danger.

The main problem in the commercial scale production of hyaluronidases from hepatopancreas is the difficulty to remove from the organism, store and transport hepatopancreas in complicated marine fishing conditions. The solution of this problem and preclinical studies on the model animals demonstrating the safety and therapeutic efficiency of the red king crab hepatopancreas hyaluronidase can replenish arsenal of preparations in treating filler complications in the near future.

4. Materials and Methods

HA products: Revofil Ultra (Lot RTU-13-08), Teosyal Ultra Deep (Lot TSU 110503B), Hyaluform filler deep (Lot E0102 04/19), HA sodium salt from rooster comb (H5388, Sigma Aldrich). All tested fillers consist of cross-linked and free HA at different ratios, contain different amounts of HA in 1 ml (Figure A1, Teosyal Ultra Deep filler stock preparation is 25 mg HA per mL). The HA fillers were stored in a place protected from light exposure at temperature not exceeding 25 °C, prepared solutions of the HA fillers were stored at 4 °C. Hyaluronidase products: Lidase (Lot 110319) and Liporase (Lot 080521). The preparations are of animal origin; they contain different amounts of the units of activity per 1 g of dry substance (Figure A2). The hyaluronidase products were stored at 4 °C, prepared solutions of the hyaluronidases were stored at −20 °C.

The tested products of fillers and hyaluronidase were purchased at a pharmacy.

4.1. A turbidimetric method for determination of hyaluronidase activity

The analysis was conducted according to previously developed protocols with minor changes [18,19,21]. The content of the fillers was diluted by phosphate buffer (160 mM sodium hydroortophosphate•12H2O, 39 mM sodium chloride, pH 5.55 was adjusted with hydrochloric acid) up to the concentration of 2.5 mg of HA in 1 mL. An aliquot of the hyaluronidase solution was added to 0.2 mL of the HA solution, the reaction mixture was thoroughly mixed and incubated at 37 °C. At time points during incubation, 10–35 µL aliquots were taken, then immediately mixed up with the phosphate buffer (up to 0.5 mL) and 2.5 mL of acid albumin (24 mM sodium acetate, 79 mM acetic acid, 50 mM sodium chloride, 1% albumin, pH 3.5). The mixture was thoroughly mixed and incubated at room temperature for 50 min. Optical density was then measured at 600 nm on a Hitachi U-2000 spectrophotometer.

4.2. Preparation of a homogenate of hepatopancreas of the red king crab (HPC)

The HPC homogenate was prepared as follows. The red king crab Paralithodes camtschaticus was caught by the crab catching vessel from the company CJSC «Arcticservice» in the Barents Sea during crab fishing season. The hepatopancreas of several crabs was separated and immediately frozen, stored and transported at −25 °C. To the hepatopancreas thawed at room temperature in laboratory conditions, distilled water and ice were added in the ratio of 1:8:2, and stirred at low speeds for 60 min. All final mixture was placed in a separating column and left for 2 h at −14 °C. An aqueous phase of the homogenate was filtrated on a hollow fiber ultrafiltration module «AP-3-300» (Scientific-Production Complex (SPC) «Biotest», Russia). The sterile solution had a dark amber color. This solution was concentrated on a hollow fiber ultrafiltration module «AP-3-1» (SPC «Biotest», Russia). An aliquot of the final product (a concentrated product) was stored at −20 °C. Protein concentration was determined with a colorimetric method using the biuret reagent [22]. Albumin («Amresco», USA, Lot 0205C125) served as calibration protein.

4.3. Decontamination of the homogenate of HPC

Ammonium sulfate up to 50% saturation was added to 1 mL of the homogenate to purify the obtained preparation from impurities. Decontamination procedure was carried out at 4 °C, ammonium
sulfate was slowly added while stirring. After centrifugal precipitation for 10 min at 10,000 g and 4 °C, the precipitate was solved by 0.5 mL of phosphate buffer, and dialysis was performed in the same buffer for 24 hours. The preparation obtained was centrifuged for 10 min at 10,000 g and 4 °C and passed through a 0.22 µm filter («Millipore», USA). The solution was colorless. The sample was analyzed by gel electrophoresis according to Laemmli [23].

4.4. Atomic force microscopy (AFM)

Sample preparation procedure for AFM was as follows. Buffer solution was used to bring the fillers up to 2.5 mg/mL. To prepare samples for AFM, the filler samples after incubation for 40 min at 37 °C were used. For hydrolysates, an equal volume of the sample was taken from the reaction mixture after 5, 40 and 120 min of incubation at 37 °C. Also, we analyzed the decontaminated preparation of the HPC homogenate 21 times diluted by phosphate buffer after its incubation for 40 min at 37 °C. For preparing samples for AFM, 2 µL of the sample were taken and transferred to freshly cleaved mica and incubated for 5 min. The sample was then washed twice in a drop of distilled water deionized by a type I Milli-Q system for 30 sec and dried in the air. AFM imaging was performed with AFM Integra-Vita microscope («NT-MDT», Russia) in noncontact (tapping) mode in air. The typical scan rate was 0.5–1 Hz. Measurements were carried out using cantilevers NSG03 with a resonance frequency of 47–150 kHz and ensured 10 nm tip curvature radius. The processing and presentations of AFM images were performed using Nova software («NT-MDT», Russia) and Gwyddion 2.44 software (http://gwyddion.net/, Czech Republic).

4.5. Nuclear magnetic resonance (NMR) of HA-based filler and its hydrolysates

One-dimensional (1D) 1H-NMR spectra were acquired on a Bruker Avance III 600 spectrometer (The Core Facilities Centre of Institute of Theoretical and Experimental Biophysics of the RAS) operating at a frequency of 600 MHz (1H) and a probe temperature of 310 K. The samples were placed in NMR tubes with diameters of 5 mm. The pulse sequences used in the experiments were standard pulse sequences from the Bruker NMR pulse sequence library. The 1D-pulse sequence ZGPR was applied for the suppression of proton signals from water. The free induction decay (FID) was collected into 96K data points using an acquisition time of 3.42 s. The spectrum width was 24 ppm and the 90° pulse width was 16 µs, the relaxation delay was 10 s. NMR spectra were obtained sequentially with time fixation.

The sample of the filler was diluted by phosphate buffer (160 mM sodium hydroortophosphate•12H2O, 39 mM sodium chloride, pH 5.55) up to the concentration of 2.5 mg of HA in 1 mL. 30 µL of D2O and 30 µL of decontaminated preparation of the HPC homogenate was added to 600 µL of HA solution, preheated for 1 h at 37 °C. The sample was stirred, centrifuged for 1 min at 15,000 g and placed into NMR tube. Time points were begun to count after addition of HPC homogenate. After 50 spectra the sample was moved into eppendorf tube and 10 µL of 3.3156 µM solution of 3-trimethylsilyl [2,2,3,3-2H4] propionate (TSP) in D2O as a standard marker was added to the sample. The mixture was placed into NMR tube and 1H spectra were recorded. At each stage samples in eppendorf tubes were weighed with the use of «Sartorius CP-64» (d = 0.1 mg).

The control sample (600 µL of phosphate buffer, 30 µL of D2O and 30 µL of decontaminated preparation of the HPC homogenate) did not give any signal except 1H of H2O (data not shown).

The processing of the spectra and the calculation of the integrals were carried out in the Bruker TOPSPIN program.

Based on the signals of the protons of the acetyl group of HA in a free state, the concentrations of HA were calculated at each time point. The concentration was calculated relative to the known concentration of the added TSP marker. Molar concentration of HA was estimated based on the disaccharide unit of D-glucuronic acid and N-acetyl-D-glucosamine. The exponential curve was obtained by approximation and was described by the equation:

\[ C = C_0 + a \times (1 - \exp(-b \times t)) \]
\[ C_0 = 0.0411 \]
\[ a = 1.501 \]
\[ b = 0.0402 \]

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**Abbreviations**
The following abbreviations are used in this manuscript:

- HPC Hepatopancreas of the red king crab
- AFM Atomic force microscopy
- HA Hyaluronic acid
- NMR Nuclear magnetic resonance

**Appendix A**

| HA filler, manufacturer | Composition | The HA component crosslinked with a crosslinking agent selected from the group consisting of |
|-------------------------|-------------|-----------------------------------------------------------------------------------------|
| Juvederm Ultra 2, «Allergan» (USA) | 24 mg/mL of HA, 0.3% lidocaine by weight of the composition, 90-95% high-molecular-weight of HA (1–4 MDa) and low-molecular-weight (0.2–1 MDa) species. HA uncrosslinked from 89% to 96%, HA crosslinked from 4 to 11% [24,25] | 1,4-butanediol diglycidyl ether (BDDE), 1,4-bis(2,3-epoxypropoxy)butane, 1,4-bisglycidyloxybutane, 1,2-bis(2,3-epoxypropoxy)ethylene and 1-(2,3-epoxypropyl)-2,3-epoxycyclohexane, and 1,4-butanediol diglycidyl ether or combinations thereof |
| Hyaluform filler deep, «Laboratory THOSCANE» (Russia) | 25 mg/mL of HA, 2 MDa, 99.8% crosslinked HA and 0.2% uncrosslinked HA [26] | ethylene glycol diglycidyl ether, diethylene glycol diglycidyl ether, triethylene glycol diglycidyl ether, polyethylene glycol diglycidyl ether, propylene glycol diglycidyl ether, diglycidyl ether of 1,4-butanediol, diglycidyl ether of 1,6-hexanediol |
| Revofil Ultra, «Caregen» (South Korea) | 23 mg/mL of HA, Oligopeptide-72 (CG-Boostrin 1,000 ppm), Oligopeptide-50 (CG-Glamerin 300 ppm), 90% crosslinked HA, 10% uncrosslinked HA [27] | 1,3-butienglycol |
Table A2. Comparison of several hyaluronidase products

| Hyaluronidase product, manufacturer | Composition | Origin |
|------------------------------------|-------------|--------|
| Lydaze, «Microgen» (Russia)        | Hyaluronidase 1280 IU [28] | Cattle testicles |
| Longidaze, «Petrovax Pharm» (Russia) | Hyaluronidase 3000 IU. Hyaluronidase is conjugated with high-molecular weight synthetic support (40–100 kDa) with ratio enzyme to support 1:1–5 [29] | Cattle testicles |
| Liporase, «Caregen» (South Korea)  | Hyaluronidase 1500 IU | Ovine testicles |

Figure A1. The study of HA of rooster comb using turbidimetric analysis during the hydrolysis by decontaminated HPC homogenate (the ratio of the total protein of the homogenate (mg) to HA (mg) in the reaction mixture is 1:20, respectively).

Figure A2. The study of Revofil Ultra filler HA using turbidimetric analysis during the hydrolysis by Lydase and Liporase (the ratio of the total protein of the hyaluronidases (mg) to HA (mg) in the reaction mixture is 1:2, respectively).

Figure A3. SDS-PAGE electrophoresis of HPC homogenate proteins before and after decontamination: 1—protein marker (bottom-up: 14.4, 20.1, 30, 43, 67, 94 kDa), 2 and 3—bovine serum albumin, 7 µg and 0.7 µg, respectively, 4—HPC homogenate before decontamination, 5—supernatant after salting out the homogenate with ammonium sulfate to 50% saturation during decontamination, 6—HPC homogenate after decontamination.
Figure A4. AFM images of HPC homogenate diluted 21 times by phosphate buffer obtained in the tapping mode: (a)—before decontamination, (b)—after decontamination.

Figure A5. Hyaluform HA-based filler by turbidimetric analysis during incubation at 37 °C (0 min means that the HA was not warmed up before the beginning of the experiment).

Figure A6. AFM images of Revofil Ultra HA-based filler obtained in the tapping mode.
Figure A7. 2D AFM images of Hyaluform filler HA hydrolysates obtained in the tapping mode: (a)—5 min, (b)—40 min, (c)—120 min hydrolysis of Hyaluform filler HA using the decontaminated HPC homogenate.

Figure A8. AFM images of Hyaluform filler HA hydrolysates obtained in the tapping mode: (a)—5 min, (b)—40 min, (c)—120 min of hydrolysis reaction of Hyaluform filler HA using the decontaminated HPC homogenate.
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