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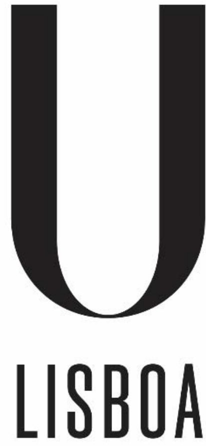

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Orientadores:

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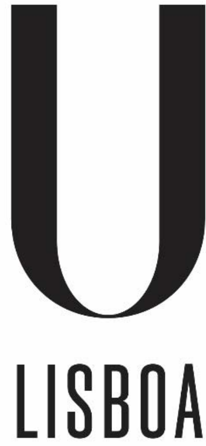

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Resumo

O ácido hialurónico é um glicosaminoglicano com capacidade de absorção de água, cuja utilização tem sido cada vez mais frequente em *fillers* injetáveis, de modo a melhorar as características estéticas da face. Com o crescente aumento da popularidade e utilização de *fillers* de ácido hialurónico na medicina estética, surgiu a necessidade de encontrar um antídoto eficaz para tratar possíveis complicações relacionadas com estes procedimentos, como por exemplo oclusões vasculares ou reações alérgicas. Neste contexto, a hialuronidase, uma enzima com capacidade de degradar o ácido hialurónico em ácido glucorónico e N-acetilglucosamina (NAG), tem sido amplamente utilizada. Esta enzima tem sido objeto de estudo crescente desde a primeira metade do século XX. Para além do seu uso na medicina estética, embora ainda *off-label*, é utilizado em diversas outras áreas da medicina. Em 1971, esta enzima foi bem documentada e classificada por Karl Meyer em três origens distintas: animal, microbiana e de moluscos, com base não só na sua estrutura como também na sua atividade enzimática. Após vários estudos, foi desenvolvida uma enzima com maior atividade, criada por engenharia recombinante, com recurso ao DNA de hamsters. Atualmente, as formulações mais frequentemente utilizadas na prática clínica são de origem animal e de origem recombinante. Apesar da importância da hialuronidase no contexto dos *fillers* de ácido hialurónico, existe uma escassez de estudos na literatura relativamente a este tema, nomeadamente na comparação da efetividade de hialuronidases de diferentes origens na degradação de ácido hialurónico. Além disso, a influência que as propriedades físicas dos *fillers* podem ter nesta degradação também necessita de maior investigação, apesar de ser um tema já mais desenvolvido. Não existe consenso no que diz respeito às concentrações de enzima a utilizar, sendo premente uma padronização para facilitar a aplicabilidade clínica destas enzimas.

Com base nestas limitações, foi identificada a necessidade de realizar um estudo que comparasse a efetividade de duas hialuronidases de diferentes origens na degradação de *fillers* injetáveis de ácido hialurónico, tendo sido esse o objetivo do estudo experimental apresentado.

Para a realização do estudo experimental, foram utilizados dois *fillers* de ácido hialurónico da marca Fillmed® (França) e duas hialuronidases distintas, uma animal da marca InstitutoBcn® (Espanha) e outra recombinante da pbserum® (Espanha), formando assim quatro grupos de estudo: ART FILLER® Universal com hialuronidase animal, ART FILLER® Universal com hialuronidase recombinante, ART FILLER® Volume com hialuronidase animal e ART FILLER® Volume com hialuronidase recombinante. A seleção de *fillers* e hialuronidases para

este estudo baseou-se no que é frequentemente utilizado em Portugal e nas opções às quais tínhamos acesso mais facilmente. A marca Fillmed® é apresentada em poucos estudos na literatura, daí haver uma necessidade acrescida do seu estudo. A nível de hialuronidases, optou-se por utilizar uma hialuronidase de origem animal devido ao extenso histórico de estudos a que esta já foi submetida ao longo dos anos, e uma hialuronidase de origem recombinante, por ser uma formulação mais recente e com boas propriedades já documentadas na literatura. A concentração utilizada de cada hialuronidase foi de 75 UI (Unidades Internacionais) para cada 0,1ml de cada *filler* (25mg/ml), o que vai de encontro à literatura previamente estudada, dentro das limitações que esta apresenta. Para chegar a esta concentração, ambas as hialuronidases foram diluídas em solução salina. A cada grupo foi adicionado um reagente, p-dimetilaminobenzaldeído, também conhecido como reagente de Ehrlich, que tem a capacidade de reagir com as cadeias de NAG, libertadas pelo ácido hialurónico aquando da sua degradação por hialuronidase, fornecendo uma cor violeta à amostra. Utilizando uma técnica colorimétrica, a quantidade de NAG libertada foi posteriormente medida através da absorbância (A) com recurso a um medidor de placas (Perkin Elmer® Inc., USA). Quanto maior a degradação do ácido hialurónico, mais cadeias de NAG livres, e assim, maior será o valor de absorbância, uma vez que haverá uma cor mais intensa do reagente. Esta medição da absorbância foi realizada em quatro momentos distintos ao longo do tempo: após 1, 6, 24 e 48 horas de incubação a 37ºC, de modo mimetizar as condições de temperatura no corpo humano, com N=5. Os resultados de absorbância foram apresentados sobre a forma de média ± desvio padrão (DP) e posteriormente analisados recorrendo a um teste não-paramétrico, após se mostrar a ausência de normalidade da amostra, através de um *software* específico de análise estatística. H0: não existem diferenças na degradação dos dois *fillers* pelas duas hialuronidases.

A utilização rigorosa deste protocolo experimental permitiu chegar a resultados significativos, onde se demonstrou que ambos os *fillers* sofreram degradação enzimática por ambas as hialuronidases (animal e recombinante). Após a análise estatística dos resultados, com recurso a um teste não-paramétrico, a hipótese nula foi rejeitada, provando que existem diferenças estatisticamente significativas na degradação dos *fillers* pelas duas hialuronidases. No entanto, ao realizar uma comparação das duas hialuronidases individualmente na degradação de cada *filler*, compreendeu-se que só se verificaram diferenças estatisticamente significativas na degradação de ART FILLER® Universal pelas hialuronidases às 24 horas (p=0,007), tempo no qual a hialuronidase recombinante se mostrou mais efetiva em comparação com a animal. No entanto, ao analisar o comportamento de ART FILLER® Volume ao longo do tempo,

constatamos uma degradação estatisticamente significativa maior pela hialuronidase recombinante às 6 horas ($p<0,001$), 24 horas ($p=0,007$) e 48 horas ($p<0,001$) quando comparado com a hialuronidase animal. Estes dados mostram que a hialuronidase recombinante apresenta maior degradação de ART FILLER® Volume logo depois das 6h. Ao realizar a comparação individual dos dois *fillers*, quando submetidos à mesma hialuronidase, apenas se observaram diferenças estatisticamente significativas à 1h, na degradação destes por hialuronidase animal ($p<0,001$), onde se observou uma maior degradação de ART FILLER® Universal. A nível de degradação pela hialuronidase recombinante, não foram demonstradas diferenças entre os *fillers*. Assim, observou-se que não existem diferenças significativas no *filler* em si que possam influenciar a sua degradação, quando comparado com o outro *filler* em análise, após 6h de incubação.

Com base nos resultados obtidos, o nosso estudo permitiu concluir que, entre todos os grupos analisados, a hialuronidase que apresenta melhores resultados ao longo do tempo é a recombinante, principalmente na degradação de ART FILLER® Volume, enquanto ART FILLER® Universal não mostrou diferenças significativas na sua degradação pelas duas enzimas na maioria dos tempos medidos. Também se observou que não há diferenças na degradação dos dois *fillers* diferentes quando submetidos à mesma hialuronidase, nem quando submetidos à de origem animal nem à de origem recombinante, a partir das 6h de incubação.

O estudo realizado contribui para a evolução do conhecimento e da literatura existente, porém, é importante destacar que existiram diversas limitações que podem ser superadas com a formulação de estudos futuros na área. Entre essas limitações encontram-se a falta de recursos, o que impossibilitou a realização de medições mais frequentes ao longo do tempo, para uma melhor compreensão do comportamento da enzima e da sua interação com o ácido hialurónico. Para além disso, tratando-se de um estudo *in vitro* há sempre condições que são difíceis de mimetizar e deve-se ter isso em consideração ao extrapolar os resultados deste estudo para a prática clínica. Muitos dos estudos presentes na literatura utilizam concentrações de hialuronidase muito elevadas, que dificilmente são extrapoladas para a prática clínica sem nenhum risco acrescido. Sugerimos que futuras pesquisas avaliem não só as duas hialuronidases mencionadas neste estudo como também analisem o seu comportamento ao longo de um maior período de tempo e com diferentes concentrações.

Abstract

Objectives: This study aimed to evaluate and compare the effectiveness of two hyaluronidases in degrading two different hyaluronic acid fillers.

Materials and methods: Four groups were tested: ART FILLER® Universal and animal hyaluronidase, ART FILLER® Universal and recombinant hyaluronidase, ART FILLER® Volume and animal hyaluronidase and ART FILLER® Volume and recombinant hyaluronidase (N=5). A reagent that reacts with N-acetylglucosamine (NAG) was added. Absorbance was measured at 585 nm after 1, 6, 24 and 48 hours of incubation at 37ºC. Results were expressed as mean and standard deviation (±SD) of absorbance and analyzed using a non-parametric test.

Results: All tested HA fillers were susceptible to degradation by hyaluronidase. Comparing the degradation of ART FILLER® Universal by the two hyaluronidases, statistically significant differences were observed at 24 hours, with recombinant hyaluronidase showing higher degradation than animal (p=0,007). For ART FILLER® Volume, recombinant hyaluronidase resulted in higher absorbance values than animal, throughout the entire time of degradation, except at 1h (6h p<0,001; 24h p=0,007; 48h p<0,001). Differences were also observed when comparing the effect of the same hyaluronidase on degrading both HA fillers. Animal hyaluronidase demonstrated greater effectiveness in degrading ART FILLER® Universal, compared to ART FILLER® Volume, particularly significant at 1h (p<0,001). However, no statistically differences in the degradation of the two HA fillers were found with recombinant hyaluronidase.

Conclusion: Recombinant hyaluronidase appeared to be the most effective overall. However, the significantly higher degradation values compared to animal hyaluronidase were only observed when paired with ART FILLER® Volume, at 6, 24 and 48 hours. Therefore, in clinical practice, recombinant hyaluronidase should be preferred when treating complications associated with prior injection of ART FILLER® Volume. When comparing the two HA fillers, no significant differences were observed, except at 1h, when animal hyaluronidase exhibited greater effectiveness in degrading ART FILLER® Universal.

Keywords: Hyaluronoglucosaminidase; Hyaluronic Acid; Enzyme Assays; Colorimetry.

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List of abbreviations

HA: Hyaluronic acid

Hyal: Hyaluronidase

USA: United States of America

FDA: Food and Drug Administration

IU: International Units

mg: Milligrams

BTH: Bovine testicular hyaluronidase

OTH: Ovine testicular hyaluronidase

BSE: Bovine spongiform encephalopathy

rHuPH20: Recombinant human PH20 hyaluronidase

DNA: Desoxyribonucleic acid

%: Percentage

PEG: Polyethylene glycol

IgG: Immunoglobulin G

IgE: Immunoglobulin E

ml: Milliliters

BEL: Belotero®

JUVX: Juvederm Ultra XC®

JUVX+: Juvederm Ultra XC Plus®

JUVV: Juvederm Voluma XC®

RESL: Restylane-L®

RESS: Restylane Silk®

RESLYFT: Restylane Lyft®

NASHA: Non-animal stabilized hyaluronic acid

H0: Null hypothesis

H1: Alternative hypothesis

µkat: Microkatal

h: Hour

Fig.: Figure

µl: Microlitre

rpm: Revolutions per minute

ºC: Degree Celsius

NaCl: Sodium chloride

g: Gram

HCl: Hydrochloric acid

DMAB: p-dimethylaminobenzaldehyde

NAG: N-acetylglucosamine

A: Absorbance

nm: Nanometre

IBM: International Business Machines Corporation®

SPSS: Statistical Package for the Social Sciences®

SD: Standard deviation

1. Introduction

Hyaluronic acid (HA) is a glycosaminoglycan composed of 2.000-25.0000 dissacharides – D-glucuronicacid and D-N-acetylglucosamine – linked by β-1,4 and β-1,3 glycosidic bonds. [1] This molecule can be found in the intercellular matrix of connective tissues of vertebrates, with emphasis on the skin, where accounts for 50% of total body HA. [2] The half-life of HA is less than a day in the skin, while in the blood it's only 3-5 minutes. [3] Among other functions, HA is known for hydrating the skin, because of its high water-binding capacity, and it also plays a role in skin aging, since it prevents cellular damage from free radicals. [2,4]

1.1. Hyaluronic acid on the perioral region

HA can be isolated from animal or bacterial sources, in order to produce HA fillers. These should have specific properties, such as biocompatibility, biodegradability, viscoelasticity and nonimmunogenicity, to be used as a cosmetic product. [2] HA achieves stability as a cosmetic filler by cross-linking the molecules with a plasticizing agent, which also helps delay its decomposition. [1] Since HA is biocompatible and has good viscoelastic properties, it has been used in cosmetic, for example, for soft tissue augmentation. HA fillers can have a long-lasting effect because HA stimulates collagen synthesis and inhibits collagen degradation. [5] The characteristics of fillers are specific from each manufacturer, and they can variate in concentration of HA, particle size, degree of cross-linking, and other characteristics. [6]

Skin aging is a complex process where HA is degrading at a certain level, and epidermis loses capacity to retain water, resulting in less skin moisture and, consequently, wrinkling, loss of face volumes, etc. HA fillers are widely use in these patients, that look for a way to improve their self-esteem. [7] When injecting HA on the perioral area is very important to have proper knowledge of the anatomy of the face, in order to avoid certain arteries, veins and glands, since an intravascular injection can occur, leading to vascular occlusion and possible necrosis. [8, 9]

1.2. Hyaluronidase as a natural enzyme

Hyaluronidase (Hyal) is an endoglycosidase, more specifically an endo-β-N-acetyl-hexosaminidase, an enzyme that can degrade HA. [1,10] Hyaluronidase works by breaking down the N-acetyl-D-glucosaminidic linkages in the HA polymer into monosaccharides. [11,12] Its

main activity is on hyaluronic acid glycosiding bonds, but it also acts on other mucopolysaccharides from connective tissues. (13)

Hyaluronidase has been known since the first half of the 20th century and research in this area has quickly increased since 1940, with emphasis on the last 20 years, where most recent discoveries on isolation, purification, application and characterization of this enzyme took part. (11) It was first discovered in bacteria but soon found to be present in many classes of organisms, including mammals, insects and even humans. The first known review of hyaluronidase was published by Duran-Reynals in 1942, and only five years later, Karl Meyer published his review, focusing on the functions and interactions between hyaluronidase and hyaluronic acid. To this day, their work continues to serve as the basis for studying hyaluronidase. (12) In the past, hyaluronidase was used in its non-purified form after extraction from mammalian testicles, resulting in a low-purity enzyme. Nowadays, mammalian hyaluronidase is purified and other hyaluronidases are used more often, in order to reduce side effects. (1)

1.2.1 Indications

Hyaluronidase has many indications, but only a few are approved by the United States of America (USA) Food and Drug Administration (FDA), including the use of hyaluronidase to intensify both the absorption and dispersion of injected drugs, enhance absorption of radiopaque agents in urography and its use in hypodermoclysis. (14) The off-label use of hyaluronidase is widespread in medicine, as it can be used to assist in local anesthesia, in different areas such as oncology and ophthalmology, and in aesthetic medicine. (14,15) In the last one mentioned, it’s often used to reverse the effects of HA fillers when there’s any complication. (15)

Despite the fact that the use of hyaluronidase in orofacial harmonization is not approved by the FDA, this enzyme is an important resource that should be present in every clinic that works with HA. Complications associated with HA fillers include vascular occlusion from intravascular injection, poor aesthetic outcomes, that can result from incorrect placement or filler migration, and delayed-onset nodules, that may appear weeks or months after injection due to various causes. Hyaluronidase is effective in treating almost all of these complications, guaranteeing a healthy and aesthetic outcome. (15)

1.2.2. Mechanism of action

After being injected into the body, hyaluronidase undergoes different reactions until the effect on HA ends. It is continuously deactivated through metabolization by anti-hyaluronidase enzymes produced by the human body. Metabolization has different rates depending on the tissue where we find hyaluronidase. For example, when injected subcutaneously, it has been observed in rodents that it has a half-time of 30 minutes. However, when in human plasma, it has a much shorter half-life, about 2 to 3 minutes. [16,17] This happens because there's a high quantity of hyaluronidase inhibitors in plasma and because of kidney and liver metabolism. Although kidney metabolism is well known, the way hyaluronidase is inactivated in the dermis and other tissues is not yet fully understood. [10] These levels of anti-hyaluronidase enzyme are not the same in everyone, since it depends, for instance, on their physical condition. Besides deactivation, hyaluronidase also undergoes degradation and diffusion, since it tends to move from higher to lower concentration areas, moving away from the original injection site. In case of ischemia, it's also common for the hyaluronidase to be diluted by swelling fluid that accumulates after leaking from capillaries. To ensure that a certain amount of hyaluronidase remains at the injection site, it's necessary to counteract this diffusion, deactivation and dilution, by progressively increasing the hyaluronidase concentration. [18]

The ability of hyaluronidases to degrade hyaluronic acid depends not only on the acid concentration but also on the cross-linking between its molecules, as well as the amount of acid. If the HA has a large amount of cross-linking in its molecules, it's harder for hyaluronidase to access the biding sites, taking a longer time to degrade the acid. [1] Hyaluronidase takes longer to dissolve larger quantities of HA. The type of HA filler will also influence its degradation. Monophasic fillers degrade slower due to their homogenous and cohesive cross-linked mixture of HA, while biphasic fillers are a heterogenous mixture of both cross-linked and non-cross-linked HA, contributing to a different degradation rate by hyaluronidase. [19]

1.2.3. Side effects

Side effects associated with hyaluronidase administration mainly include allergic reactions, with local reactions being the most common in this case. The incidence of local allergic reactions ranges from 0,05% to 0,69%, with symptoms including pain, edema and erythema. [10] However, when higher doses (200.000 IU) of hyaluronidase are administrated, the incidence of allergic reactions can increase to 31,3%. [10,20] These reactions are usually type

I or immediate hypersensitivity reactions, occurring within 1-2 hours, but type IV or delayed hypersensitivity reactions can also occur, even after 24 hours. [1] Individuals with a history of type I hypersensitivity reactions, especially bee or wasp allergy, must undergo a skin test to avoid further complications, as there's a significant risk of reactivity. While the enzyme itself is considered the direct cause of allergy, it is also possible that impurities can increase the chance of having an allergic reaction. [15]

1.2.4. Classifications

Meyer (1971) organized hyaluronidases into three different families, according to their mechanism of action, as well as their origin: [21] mammalian hyaluronidase, microbial hyaluronidase, and leech hyaluronidase.

1.3. Animal/Mammalian Hyaluronidase

Mammalian hyaluronidases are hyaluronate 4-glycanohydrolases and degrade HA through hydrolysis by cleaving the β-1,4 glycosidic bond. The best-known mammalian hyaluronidases are testicular, including bovine and ovine. Human hyaluronidase has also been studied extensively lately.

Mammalian Hyal have been used in several ways, mainly as an adjuvant to administer local anesthetics, insulin and other large molecules. [22-24] Hyaluronidase also revealed to have a role on cancer but that remains unclear to the scientific community. Some studies suggest that Hyals have an inhibitory effect and can be considered tumor suppressors, while others suggest that they have both inhibiting and facilitating effects on cancer, depending on the cellular concentration of hyaluronidase. However, Hyal can be marked and measured to determine cancer prognosis, since it appears to be a non-invasive method. [22]

Enzyme activity is measured in concentration of active hyaluronidase protein per total protein (IU/mg). There's a difference of around 17.200 IU/mg more enzyme activity for pharmaceutically prepared hyaluronidase when compared to unprocessed hyaluronidase, making it a better option. [25]

There are also different origins within mammalian hyaluronidase, and among the most studied ones are bovine testicular hyaluronidase (BTH) and ovine testicular hyaluronidase (OTH). Both have been applied in distinct medical fields. For instance, bovine hyaluronidase

has been used in ophthalmology, as an adjuvant of anesthesia, in dermatology and in aesthetic medicine. It consists of an endo-glycanohydrolase that degrades HA, chondroitin, chondroitin-4- and -6-sulphate and dermatan sulphate. (11,25,26) The major problem with using BTH is the risk of bovine spongiform encephalopathy (BSE), justifying why several companies have stopped producing it. When we talk about OTH, there are no significant differences in HA degradation, compared to BTH. It has higher purity and there's no risk of BSE, which makes it a valid choice for daily clinical use. (25,27)

When hyaluronidase started being used in medicine, mammalian hyaluronidase, specifically, bovine hyaluronidase, was the preferred one. (28) Nowadays, it's only used when purified, to reduce the risk of an immunological reaction. (29) Some examples of available animal hyaluronidases are VITRASE® (USA) or InstituteBCN® (Spain). More studies are needed to understand the connection between mammalian hyaluronidase structure and its function. (22,28) Future studies on mammalian hyaluronidase must also search for the mechanism of those enzymes in certain diseases and establish a protocol for their proper use. (22)

1.3.1. Recombinant Hyaluronidase

Recombinant engineering has enabled the production of human recombinant hyaluronidase, with the main one being rHuPH20, a more recent work on this area. rHuPH20 is produced using Chinese Hamster Ovary cells with DNA plasmid encoding PH20, which is subsequently purified to achieve almost 99% purity. Its half-life, shorter than 30 minutes, makes it difficult to be detected in plasma after injection. (25)

The use of rHuPH20 has become important for subcutaneous application of various drugs such as morphine, insulin, antibiotics or immunoglobulins. When injected with recombinant hyaluronidase, an increased speed of drug absorption is observed. (25,30)

Recently, PEGylated rHuPH20 (PEGPH20) has been used in combination with chemotherapeutic agents for oncologic therapy of hyaluronic acid-high advanced pancreatic cancer. In this disease, the tumor produces high levels of hyaluronan, which makes penetration of high doses of chemotherapeutic drugs on the tumor difficult. PEGPH20 can degrade HA and enable the drugs to reach cytotoxic concentrations on the tumor. (31) rHuPH20 is also being studied for infusion along with immunoglobulin, as mentioned previously. Subcutaneous administration of rHuPH20 together with IgG enables hyaluronidase to degrade HA of the extracellular matrix, allowing IgG to easily reach the systemic circulation. (17)

This purified human hyaluronidase is approved by FDA and exhibits significantly higher hyaluronidase activity per protein (approximately 120,000 IU/mg) when compared to mammalian or microbial hyaluronidase. Since there's a higher level of purity in this hyaluronidase compared to mammalian, there's a lower risk of immunogenic reactions and the safety of using hyaluronidase increases. [25] Some examples of available recombinant hyaluronidases include HYLENEX® (USA) and pbserum® (Spain).

1.4. Microbial Hyaluronidase

Microbial hyaluronidases, also known as bacterial lyases, are hyaluronate lyases that don't degrade HA through hydrolysis, but through β-elimination, producing unsaturated disaccharides as the main product: 2-acetamido-2-deoxy-3-O-(-D-gluco-4-enepyranosyluronicacid)-D-glucose. [10,11,28] Different microbial hyaluronidases degrade HA by initial endolytic cuts followed by an exolytic cleavage of one disaccharide at a time, but this mechanism of action still needs further investigation to be understood. [10] This enzyme can be found in different microorganisms, such as *Streptococcus*, *Streptomyces*, *Clostridium* and *Micrococcus*. [10,11]

Microbial hyaluronidase plays a major role in diseases, contributing to the virulence of bacteria by facilitating adhesion, invasion and tissue penetration. Besides that, it also works as a smoothing factor for diffusion of other components in the venom. There are molecules such as flavonoids, saponins or some polysaccharides that can prevent infection by inhibiting hyaluronidase but they're not specific for hyaluronidase and the existence of specific inhibitors is still unknown. [32-34] Hyaluronidase is produced by pathogenic bacteria and degrades HA present in connective tissues, particularly on the skin, allowing the pathogen to penetrate and infect the body. [34]

This kind of hyaluronidase has an advantage over mammalian ones because it's unlimited and purer, resulting in a lower chance of developing an allergic reaction. [10,35] Its different degradation mechanism in the beginning and in the end of the degradation process makes it a unique enzyme. [35]

1.5. Other hyaluronidases

Although mammalian, microbial and recombinant hyaluronidases are well-known, there are other types worth mentioning, such as venom, fungi and leech hyaluronidase.

Hyaluronidase activity has been found in different animal venoms, including bees, snakes, spiders, scorpions, lizards, wasps, caterpillars, stonefish and hornets. [36] Hyaluronidase in animal venom appears to be crucial for spreading toxins from the injection site to the systemic circulation. For this to happen, hyaluronidase works as a spreading factor, degrading HA from the extracellular matrix of soft connective tissues and increasing venom diffusion. It is an important allergen of several species of scorpions, bees, hornets and wasps, which can initiate an IgE-mediated anaphylactic reaction in humans. Therefore, it is crucial to study these implications *in vivo* to better understand the consequences of venom hyaluronidase on envenomation. [11,36,37]

Leech hyaluronidases are hyaluronate 3-glycanohydrolases and are present in the salivary glands of leeches and hookworms. They have the ability to degrade HA by cleaving the β-1,3 glycosidic bond, producing tetra- and hexasaccharide end products. Despite its content being only 1/10 of bovine testicular hyaluronidase, leech hyaluronidase has a higher activity. In general, it's a poorly characterized enzyme with further need of investigation. [11,38,39]

Furthermore, hyaluronidase activity can be found in some species of fungi, such as *Candida*. Hyaluronidase and chondroitin sulphatase are important pathogenic factors for oral infectious diseases, and are both produced by *C. albicans*, one of the main pathogens that cause oral disease in humans. However, the role of hyaluronidase and chondroitin sulphatase in the pathogenesis of candidiasis is still unknown. Fungal hyaluronidase is an area that remains undiscovered but has the potential to enhance our understanding of opportunistic infections. [21,40]

1.6. Hyaluronidase quantification

Although some studies quantify hyaluronidase and its dose and time dependency to degrade HA [41,42], there's a lack of studies that quantify and compare different types of hyaluronidases. This is likely due to the absence of strict guidelines about its use, although hyaluronidase is widely used in various medical fields worldwide. [10] In the literature, hyaluronidase quantity to degrade HA fillers varies from 1.5 to 300 units, a huge unit span. [43] This range is normal, since the dose depends on the desired results and the initial HA injection

dose. For example, a dose of 5-10 IU of hyaluronidase seems to be enough for allergic reactions, while to remove hyaluronic acid bumps, a lower dose of 1,5-3 IU has been sufficient in some cases, but in others, this dose can go up to 10 IU and repeated weeks later if necessary. [44,45] Table 1 presents some of the literature's findings on hyaluronidase quantification, including case reports, clinical trials and literature reviews.

Table 1: Review of the literature: Evaluation of different hyaluronidases and HA fillers with different concentrations in order to quantify hyaluronidase dose needed to degrade a certain dose of HA.

Author	Year	HA	HYAL	Results	Conclusions
Juhász *et al.*	2017	28 injections of 0,2ml each. Different brands of HA fillers were used. From BELOTERO®, Belotero Balance (BEL) was used. From JUVÉDERM®, Juvederm Ultra XC (JUVX), Juvederm Ultra XC Plus (JUVX+) and Juvederm Ultra Voluma XC (JUVV) and from Restylane®, Restylane-L (RESL), Restylane Silk (RESS), and Restylane Lyft (RESLYFT) were the chosen ones. (4 injections/filler)	Low dose of 20 IU in one injection site. High dose of 40 IU in one injection site. 0,2ml of saline in one injection site. One control injection site.	Decrease palpation scale on days 1, 2, 3, 4, 7 and 14 postinjection of 20 and 40IU of hyaluronidase. HA fillers JUVX+ and JUVV were the only ones that showed remarkable differences in volume between 20 and 40 IU of hyaluronidase. JUVX+ and RESL were the slowest to degrade and BEL the fastest one.	Lower and higher concentrations of hyaluronidase are equally effective on degrading HA. It should be used 20 IU of hyaluronidase for every 4-6mg of HA.

Casabona *et al.*	2018	Injection of 0,1ml and 0,2ml of each HA: Juvéderm Volbella XC® (15mg/ml), Juvéderm Voluma XC® (20mg/ml), Juvéderm Ultra Plus® (24mg/ml), Belotero Volume® (25,5mg/ml) and Belotero Balance® (22,5mg/ml).	Injection of each hyaluronidase into each HA filler – on both 0,1ml and 0,2ml injections: 4 IU Vitrase® (ovine), 4IU Hylenex® (human recombinant), 4 IU Hylase Dessau® (bovine), 4 IU Hyaluronidase 2000® (bovine) and 4 IU Reductonidasa® (bovine).	Vitrase® seemed to be the most effective, in general. Belotero Balance® injections were totally degraded by Reductonidase® in less than 2 minutes. Juvéderm Voluma XC®, on the other hand, took around 16 minutes to be dissolved by Hylenex®.	Particle size, concentration, cross-linking and degree of hydration of HA makes the hyaluronidase action different on each specific filler. There are also differences between hyaluronidases according to its origin.
Vartanian *et al.*	2005	Three injections of 0,2ml of non-animal stabilized hyaluronic acid (NASHA).	3-5 days after HA injection, hyaluronidase was injected. 0,4ml in each site, of 75 IU/ml hyaluronidase (30 IU), 50 IU/ml (20 IU) hyaluronidase or 25 IU/ml (10 IU) hyaluronidase.	The dose dependency wasn't statistically significant but some changes were seen both numerically and graphically. On days 4-7 the high-dose hyaluronidase visibly degraded all HA while the medium-dose only reached that by the second week and the low-dose group didn't reach complete degradation at all.	There's a dose dependency when using hyaluronidase to solve HA fillers complications.

Menon *et al.*	2010	Injection of 0,4ml of Juvederm Ultra® (cross linked non animal HA) on both sides.	Injection of 0,2ml (3 IU) of Hynidase® (ovine hyaluronidase) on both sides.	After two days there was a complete resolution on the left side and 60% improvement on the right side, that, 2 days after another 1.5 IU injection of Hynidase®, was completely resolved.	Low doses of 1.5-3 IU of hyaluronidase can be effective, so it's preferable to start with lower doses. This is a case report so it's not possible to accept definite conclusions.
Cohen *et al.*	2015	This is a review of the literature available about the use of hyaluronidase. It concludes that the dosage of hyaluronidase depends both on the clinical context and the quantity of HA previously administered. For nodules of periorbital, perioral and nasal regions we should start the treatment with 5-15 IU of hyaluronidase. For more delicate areas 1.5-3 IU are indicated. For skin necrosis or ischemia, 30-75 IU of hyaluronidase should be administered.			

1.7. Considerations regarding hyaluronidase

Mammalian hyaluronidase is the most extensively studied, alongside microbial hyaluronidase. However, the newer formulations of recombinant hyaluronidase have shown promising results in various medical fields and its use may increase in cosmetic medicine. Currently, there's a lack of scientific evidence regarding the enzyme activity of different hyaluronidases in degrading HA, including any variations in concentration required to degrade the same quantity of HA and the time needed for this degradation. This study will focus on comparing two available hyaluronidases to provide essential information for daily clinical practice, enabling professionals to know the appropriate dose of hyaluronidase to use in the event of complications with HA injections.

2. Objectives

The aim of this study is to evaluate and compare the effectiveness of animal and recombinant hyaluronidases in the degradation of two different injectable reticulated HA fillers by a colorimetric technique.

The null hypothesis (H0) is that there's no difference in effectiveness between animal and recombinant hyaluronidase when degrading HA fillers.

The alternative hypothesis (H1) is that there's a difference in effectiveness between animal and recombinant hyaluronidase when degrading HA fillers.

3. Materials and methods

An experimental study was conducted in Faculdade de Medicina Dentária da Universidade de Lisboa. In this study we aspired to quantify the degradation of each HA filler over time, using two different hyaluronidases.

Hyaluronidase interacts with HA by breaking down the N-acetyl-D-glucosaminidic linkages in the HA polymer into monosaccharides – glucuronic acid and N-acetylglucosamine (NAG). On colorimetric assays, the reaction between a reagent and NAG will result in the production of a violet color. When there's a greater enzymatic degradation of HA, there's a higher release of NAG, enabling the reagent to interact with it and produce the violet color. This means that the greater the degradation, the more intense the color will be, and consequently, we'll have higher absorbance values. [46,47]

Four groups were evaluated: ART FILLER® Universal and animal hyaluronidase, ART FILLER® Universal and recombinant hyaluronidase, ART FILLER® Volume and animal hyaluronidase and ART FILLER® Volume and recombinant hyaluronidase (N=5).

For the control groups, we had a positive control group, consisting of a filler, hyaluronidase and no reagent, as well as a negative control group, with hyaluronidase or HA separate, which served to control the NAG identification technique.

HA fillers ART FILLER® Universal and ART FILLER® Volume (FillMed®, France), both have a HA concentration of 25mg/ml and 0,3% lidocaine on its composition.

Animal hyaluronidase was obtained from InstitutoBcn® (Spain). Each vial contains 1500 IU of powder. Recombinant hyaluronidase was purchased from pbserum® (Spain), also with 25 μkat (1500 IU) in each vial.

0,1ml of each HA filler – ART FILLER® Universal and ART FILLER® Volume – were placed in Eppendorf® tubes with 2ml capacity, previously weighted when empty. We weighted the tubes with the HA fillers and they were then centrifugated for 5 minutes at 1890rpm in a Costar® Mini Centrifuge (USA). The tubes were then placed in an incubator (Memmert®, Germany) at 37ºC for 10 minutes.

In order to obtain 75 IU in every 50µl of solution, animal and recombinant hyaluronidase powder were both diluted in 1ml of NaCl (0,9%). The saline solution was prepared by adding 0,9g of NaCl, purchased from Sigma-Aldrich® (USA), to 100ml of distilled water. We removed the tubes with HA from the incubator, added 50µl of each animal or recombinant hyaluronidase, according to test groups and control groups. After hyaluronidase addition all tubes were incubated at 37ºC. For potassium tetraborate preparation, a bottle of 500g of potassium tetraborate tetrahydrate was purchased from Sigma-Aldrich® (USA), and 24,4g were taken from it and added to 100ml of distilled water. The pH of that solution was 9.95 and the optimal pH for this experiment would be 9.1, so we used hydrochloric acid (HCl) to stabilize the pH.

Potassium tetraborate was added at different times of incubation (1h, 6h, 24h and 48h) on each group, to stop the reaction. All groups were measured after 1h, 6h, 24h and 48h of incubation. Figure 1 shows the representation of these groups.

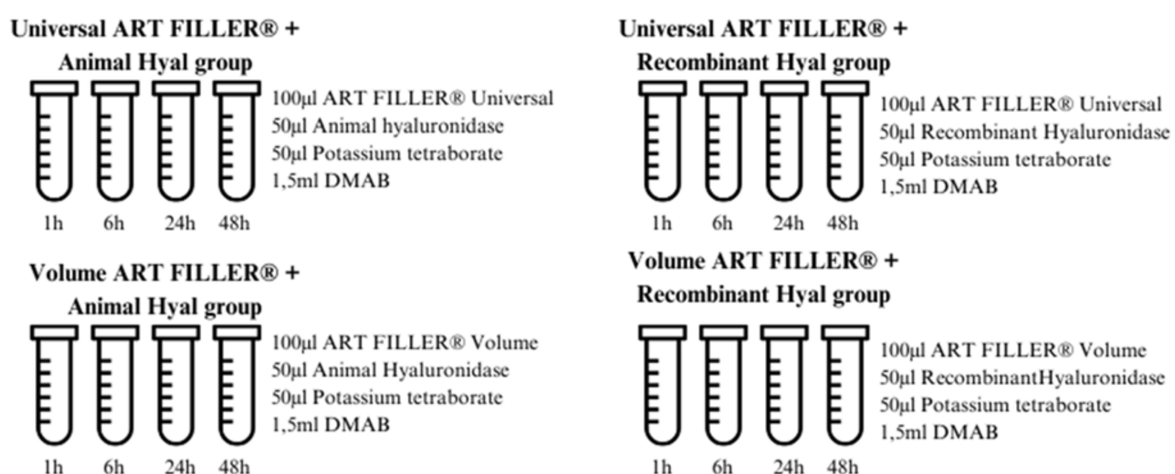

Fig. 1: Representation of the experimental groups with the specific content present in each tube.

After potassium tetraborate addition, the tubes were placed in the vortex (VELP Scientifica®, Italy) and then on a 100ºC bath for 3 minutes, in a waterbath (Köttermann®,

Germany). The tubes were cooled down to temperature room, in order to add 1,5ml of p-dimethylaminobenzaldehyde (DMAB) to each tube (except on the negative control group).

For the DMAB reagent preparation, we started by adding 25ml of HCl to 125ml of acetic acid from Sigma-Aldrich® (USA), creating our stock solution. 10g of p-dimethylaminobenzaldehyde (DMAB or Ehlrich's reagent) purchased from Sigma-Aldrich® (USA) were weighted, using a scale (Mettler® Toledo, USA), and added to 100ml of acetic acid solution. Immediately before use, this solution was diluted again in a proportion of 1:10, where 5ml of DMAB with acetic acid were added to 45ml of stock solution.

After adding the reagent, the tubes were placed in the vortex and then incubated again at 37ºC for 20 minutes, to develop a violet color according to the NAG content in each tube. Then, they were centrifugated for 15 minutes at 1890 rpm.

In order to measure absorbance (A), 200µl of each tube were placed in five different spots of a 96-well-spot (Corning®, USA), and it was read at 585 nm, with a VICTOR Nivo microplate reader (Perkin Elmer® Inc., USA).

Statistical analysis was performed using the 28th version of the IBM® SPSS® Statistics software (Chicago, IL, USA). Shapiro-Wilk normality test was performed, as well as Kolmogorov-Smirnov test. The groups did not follow a normal distribution, so it was performed a non-parametric test, Kruskal-Wallis test, followed by a comparison of groups by a pairwise method. Differences were considered statistically significant at p-value $\leq 0,05$. Results were reported in the form of mean ± standard deviation (SD).

4. Results

Our samples were measured with N=5 and the mean value (± SD) of these measurements are present on Table 2. The negative control group, which include separate samples of hyaluronidase and HA, showed no absorbance values, indicating no degradation. This control group was used to validate the NAG identification technique.

Table 2: Mean value (± SD) of absorbance (A), measured at 585 nm, of the four groups.

Time (h)	Absorbance (A)			
	Universal ART FILLER® with Animal Hyal	Universal ART FILLER® with Recombinant Hyal	Volume ART FILLER® with Animal Hyal	Volume ART FILLER® with Recombinant Hyal
1h	0,0732 ± 0,0074	0,0592 ± 0,0012	0,0526 ± 0,0012	0,0540 ± 0,0019
6h	0,1284 ± 0,0014	0,1394 ± 0,0014	0,1120 ± 0,0011	0,1504 ± 0,0016
24h	0,2350 ± 0,0048	0,2610 ± 0,0031	0,1984 ± 0,0025	0,2506 ± 0,0020
48h	0,3032 ± 0,0021	0,3174 ± 0,0022	0,2582 ± 0,0070	0,3274 ± 0,0040

Every HA filler that was tested showed a susceptibility to degradation by hyaluronidase. In fact, when each of the examined samples was exposed to hyaluronidase, there was an observable change in absorbance (A) over time, which means, as explained before, that there is a higher NAG release. Some differences can be seen on HA degradation as we examine the samples over time.

The Kruskal-Wallis test had $p \leq 0,05$, rejecting the null hypothesis and proving that there are statistically significant differences between the degradation of ART FILLER® by hyaluronidases at every time of measurement. However, after analyzing the group comparison tables, we noticed that this doesn't apply to every sample.

Although animal hyaluronidase exhibited higher degradation of ART FILLER® Universal within the first hour, when compared to recombinant hyaluronidase, the degradation rate by animal hyaluronidase started to slow down after that. In contrast, recombinant hyaluronidase showed effective degradation of ART FILLER® Universal, maintaining higher absorbance values at 6, 24 and 48 hours (Figure 2). However, the differences between the effectiveness of animal and recombinant hyaluronidase were only statistically significant at 24 hours, where recombinant showed statistically significant higher degradation than animal ($p=0,007$).

For ART FILLER® Volume, recombinant hyaluronidase resulted in higher absorbance values than animal, throughout the entire time of degradation, except on the first hour (6h p<0,001; 24h p=0,007; 48h p<0,001) (Figure 3).

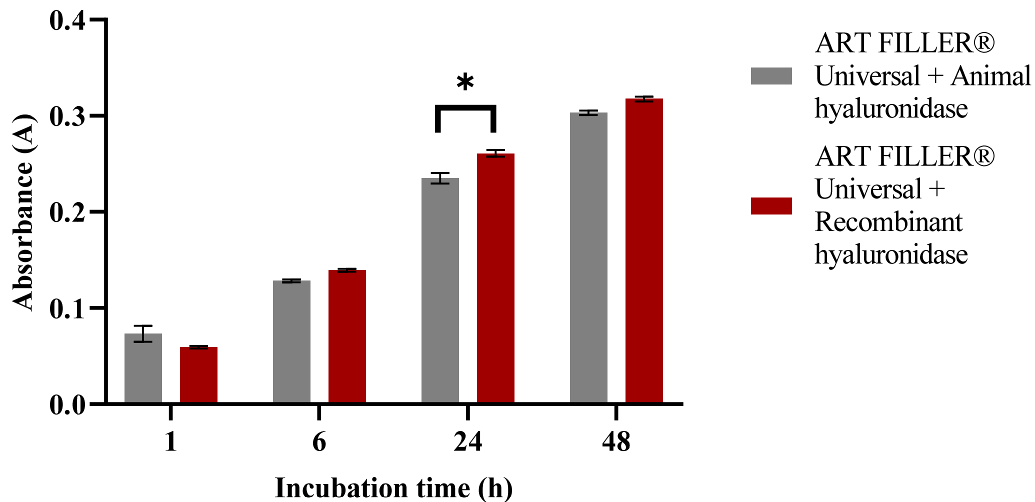

Fig. 2: Bar graph showing the mean values ± SD of absorbance, that represent the quantity of NAG release after ART FILLER® Universal degradation by animal and recombinant hyaluronidase over time (N=5). Absorbance was measured at 585 nm. *P<0,05.

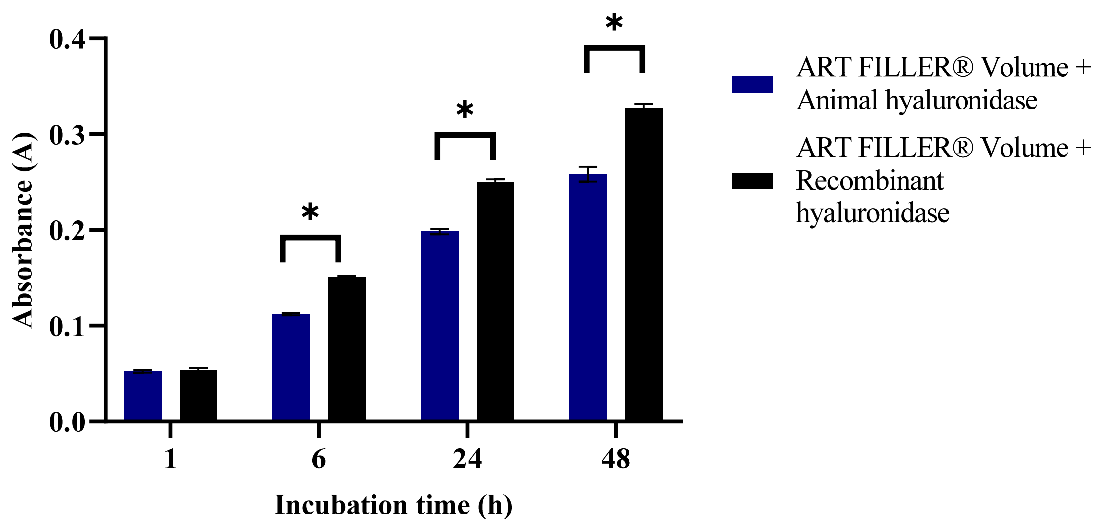

Fig. 3: Bar graph showing the mean values ± SD of absorbance, that represent the quantity of NAG release after ART FILLER® Volume degradation by animal and recombinant hyaluronidase over time (N=5). Absorbance was measured at 585 nm. *P<0,05.

Differences can be observed when analyzing the effect of the same hyaluronidase on degrading both HA fillers (Figures 4 and 5). Animal Hyal showed great effectiveness in degrading ART FILLER® Universal, compared to ART FILLER® Volume, indicating that the properties of the filler can influence its degradation. This difference is only significant at 1h, where ART FILLER® Universal degradation by animal hyaluronidase is statistically significantly higher than ART FILLER® Volume degradation by the same Hyal (p<0,001).

This aspect is not apparent when analyzing recombinant hyaluronidase, where there were no statistically differences in the degradation of the two HA fillers, and Figure 5 shows how similar the values are for both of them.

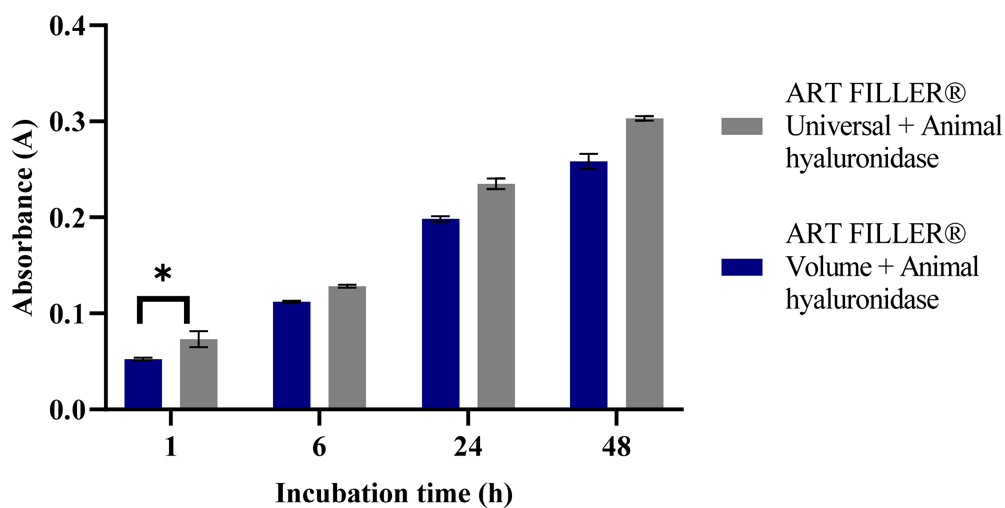

Fig. 5: Bar graph showing the mean values ± SD of absorbance, that represent the quantity of NAG release after ART FILLER® Universal and ART FILLER® Volume degradation by animal hyaluronidase over time (N=5). Absorbance was measured at 585 nm. *P<0,05.

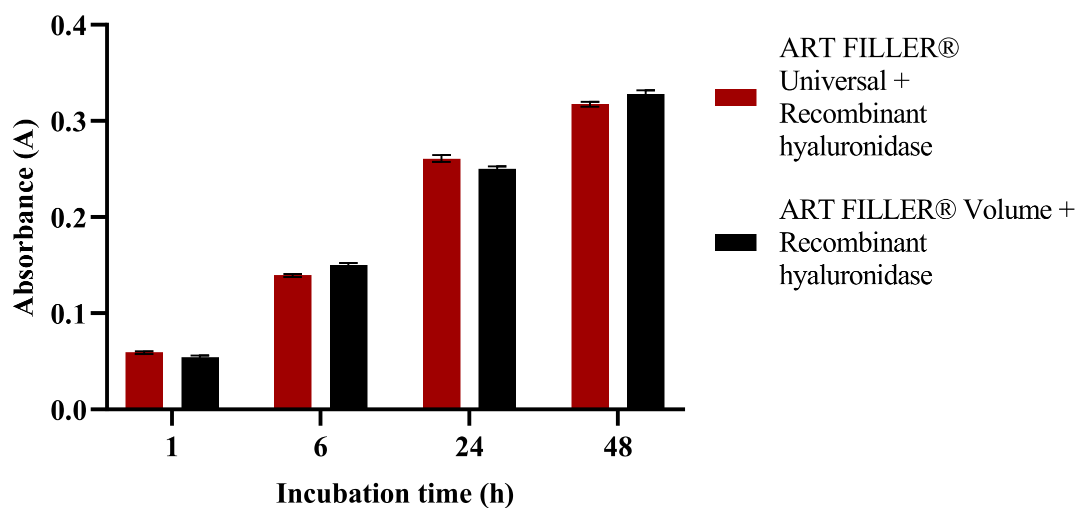

Fig. 4: Bar graph showing the mean values ± SD of absorbance, that represent the quantity of NAG release after ART FILLER® Universal and ART FILLER® Volume degradation by recombinant hyaluronidase over time (N=5). Absorbance was measured at 585 nm.

When analyzing each specific time point, it was observed that at 1h, the group ART FILLER® Universal and animal hyaluronidase exhibited higher absorbance values. This group showed to have statistically significant higher values than ART FILLER® Volume with animal hyaluronidase (p>0,001), and higher values than ART FILLER® Volume with recombinant hyaluronidase (p=0,02). ART FILLER® Universal with recombinant hyaluronidase also showed to have statistically significant higher values than ART FILLER® Volume with animal hyaluronidase (p=0,023).

At 6h and 48h ART FILLER® Volume and recombinant hyaluronidase displayed higher absorbance values. This group showed statistically significant differences when compared with ART FILLER® Volume and animal hyaluronidase (p<0,001) and ART FILLER® Universal and animal hyaluronidase (p=0,007). Besides that, ART FILLER® Universal degradation by recombinant hyaluronidase was statistically significantly higher than ART FILLER® Volume by animal hyaluronidase (p=0,007).

At 24h, the group ART FILLER® Universal and recombinant hyaluronidase showed statistically significant higher values of degradation than ART FILLER® Universal and animal hyaluronidase (p=0,007), as well as than ART FILLER® Volume and animal hyaluronidase (p<0,001). Besides that, ART FILLER® Volume degradation by recombinant hyaluronidase was statistically significantly higher than ART FILLER® Volume degradation by animal hyaluronidase (p=0,007).

Over the course of 6, 24 and 48 hours, the degradation of ART FILLER® Volume by animal hyaluronidase appeared to be the less effective compared to the other groups. Recombinant hyaluronidase might take longer to achieve high rates of degradation of both fillers but its values of degradation are higher than the animal ones (Figure 6).

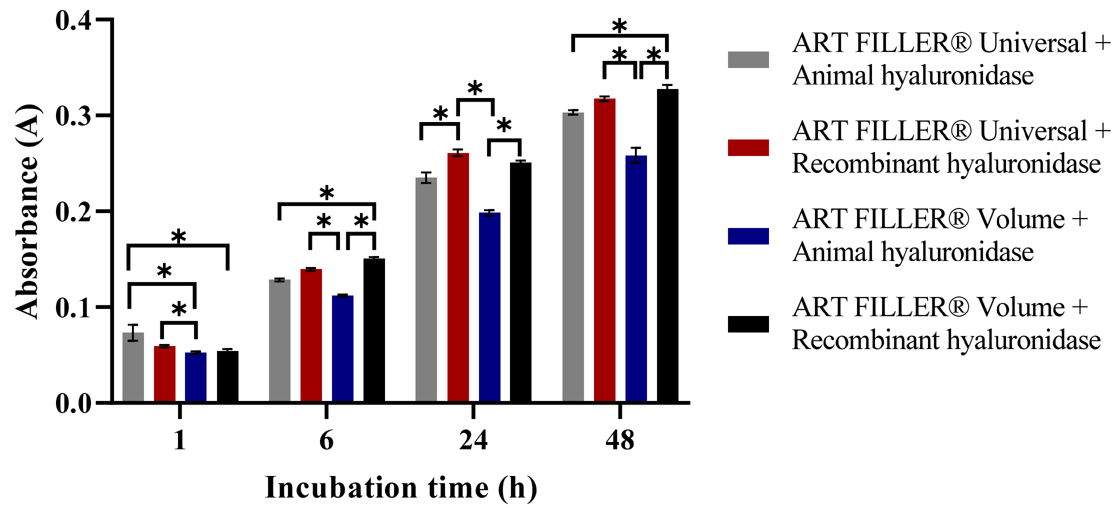

Fig. 6: Bar graph showing the mean values ± SD of absorbance, that represent the quantity of NAG release after ART FILLER® Universal and ART FILLER® Volume degradation by animal and recombinant hyaluronidase over time (N=5). Absorbance was measured at 585 nm. *P<0,05.

5. Discussion

Our study measured the degradation of two hyaluronic acid fillers - ART FILLER® Universal and ART FILLER® Volume – by two hyaluronidases – animal and recombinant – using a colorimetric method. Both HA fillers appeared to be degraded by the hyaluronidases but showed different values of absorbance over time.

There are different brands of HA and hyaluronidase available, but we used a specific brand of HA fillers called FillMed® Laboratoires (France). It is widely use in Portugal, but has been less studied than other brands such as Restylane® by Galderma (Switzerland) or Juvederm® (USA), which are frequently mentioned in the literature. FillMed® has a range of HA fillers that are used according to the objectives of both patients and physicians. In this specific case, we used ART FILLER® Universal, which is clinically used to correct medium to deep wrinkles and enhance lip augmentation, and ART FILLER® Volume, designed to create and restore skin volume. We selected ART FILLER® Universal due to its wide range of applications, as it can be used in various areas of the skin, and ART FILLER® Volume for its higher crosslinking, which enabled us to investigate whether the properties of the filler impact its degradation.

Regarding hyaluronidase, we used two different brands: InstituteBCN® (Spain) for animal hyaluronidase and pbserum® (Spain) for recombinant hyaluronidase. InstituteBCN® does not provide specific information about the recommended concentration of hyaluronidase use in each volume of injected HA, it's only available on their website that it should be diluted by adding 8ml of deionized water. On the other hand, pbserum® provides guidance on the volume of hyaluronidase that should be applied at each point of HA injection. For HA concentration of 25mg/ml, like what happens on the fillers we previously mentioned (ART FILLER® Universal and ART FILLER® Volume from FillMed®), 0,15ml-0,2ml of recombinant hyaluronidase should be used in each quadrant of the lips, if we aspire to dissolve all HA present there. In this specific study, we decided not to follow the manufacturer's recommendations for diluting animal hyaluronidase, since our aim was to have the same concentration of both hyaluronidases in order to accurately compare their effects on HA. Therefore, we also diluted animal hyaluronidase in 1ml of NaCl (0,9%) and achieved a concentration of 75 IU in every 50µl of each hyaluronidase used.

These two types of hyaluronidases, animal and recombinant, are commonly used in aesthetic medicine, and each has specific reasons for being our preferred choice. Despite animal

hyaluronidase being more associated with allergic reactions, it has been studied since 1928, providing a wealth of research and information about its functions and consequences. On the other hand, recombinant hyaluronidase is a newer formulation, with fewer published clinical trials and other studies. However, it is purer, resulting in fewer allergic reactions and higher safety levels. [25,28] Another important factor to consider is the price of both of these hyaluronidases: while animal hyaluronidase formulations are more affordable, recombinant hyaluronidase can be very expensive, which is significant for many patients.

While there aren't many studies comparing the degradation capability between two hyaluronidases, some of the existing ones indicate that there are no significant differences between HA degradation by animal and recombinant hyaluronidase, like Rao *et al*. In this case, VITRASE® (ovine hyaluronidase) and Hylenex® (recombinant hyaluronidase) were used with various HA fillers (Belotero®, Restylane® and Juvederm®) and its degradation was measured. They concluded that the degradation of fillers followed a specific order, with higher degradation of Restylane®, followed by Juvederm® and finally Belotero®. However, there was no significant difference in degradation by animal and by recombinant hyaluronidase. [48] In our study, there was only a difference between hyaluronidases on degrading ART FILLER® Volume overtime, while for ART FILLER® Universal this difference was only significant at 24h. Nevertheless, this study used different formulations of HA fillers, which can explain the difference of results compared to our study. However, there are no studies available testing the same HA fillers and hyaluronidases used in our study.

In our study, recombinant hyaluronidase exhibited higher enzymatic activity and degraded more ART FILLER® Volume, but it took more time to achieve this high level of degradation, since this was only relatable for 6h ($p<0{,}001$), 24h ($p=0{,}007$) and 48h ($p<0{,}001$). This makes recombinant hyaluronidase a suitable option for non-urgent situation when previously injected with ART FILLER® Volume. These findings are supported by the existing literature, which indicates that recombinant hyaluronidase has a higher enzymatic activity per protein compared to animal hyaluronidase. [25] On the first hour measurement in our experiment, no significant differences were noticed in enzymatic activity.

HA fillers are stabilized through a crosslinking process during their production. This crosslinking contributes to the clinical effects and longevity of the product in the skin by delaying its decomposition and making it more difficult for hyaluronidase to access the binding sites. [1,49] Crosslinking, along with other filler properties, such as particle size, HA

concentration and degree of hydration, not only affects the durability of the product but also its susceptibility to degradation by hyaluronidase. [42] Crosslinking appears to be the main property influencing the degradation of fillers. Therefore, a filler with more crosslinking and higher concentration of HA will require a longer time for proper degradation, compared to a filler with lower crosslinking. [27,50] According to the manufacturer, ART FILLER® Volume has a higher crosslinking rate and is more reticulated than ART FILLER® Universal, suggesting that it would be expected to have a lower rate of degradation. Our results indicated that there are no statistically significant differences in the degradation of ART FILLER® Universal and ART FILLER® Volume by recombinant hyaluronidase over time. With animal hyaluronidase, there are statistically significant differences at 1h, where ART FILLER® Universal exhibited greater degradation compared to ART FILLER® Volume ($p<0,001$), showing than in urgent situations, where ART FILLER® Universal was previously injected, animal hyaluronidase can be a suitable option to degrade this filler. Since the studies presented before use mammalian hyaluronidases, similar to most studies in the literature, comparing these two types of hyaluronidases provides important information for clinical decision-making. These findings suggest that properties of the fillers can influence its degradation by the two hyaluronidases initially (1h) but not over time (Fig. 4 and Fig. 5).

Casabona *et al*. conducted a study comparing the degradation of five different HA fillers from different brands, using five different hyaluronidases, also from different brands. They observed that, while ovine hyaluronidase degraded the products faster than recombinant hyaluronidase, this observation only applies to a specific brand, since there are other mammalian hyaluronidases, such as bovine hyaluronidases, from other brands, that were slower in degrading HA than the recombinant one. [42] In this case, either the origin or the brand specific formulations can justify this. In our study, we only have hyaluronidase available from two different brands: InstitutoBcn® for animal hyaluronidase and pbserum® for recombinant. Therefore, we cannot draw conclusions regarding the extent of degradation between different brands, since the characteristics of fillers and hyaluronidase can have some differences.

There have been clinical cases and other studies discussing the quantity of hyaluronidase required to degrade HA fillers, but this quantity varies significantly depending on the area being treated. For instance, it’s estimated that 15-30IU of hyaluronidase are sufficient to address HA problems in the nasal and perioral area, while for the periorbital area 30IU are needed. Smaller doses ranging from 1,5-15IU would be enough to dissolve HA in the infraorbital area and lower eyelid. [44,45] However, these existent studies use mostly animal hyaluronidase and not

recombinant hyaluronidase and some cases don't specify the quantity of HA previously injected. According to our study, for animal hyaluronidase we can hypothesize that it degraded most of both HA fillers, since it began to enter a plateau after 24h of incubation, and 75 IU can be enough to degrade 0,1ml of HA (25mg/ml). Recombinant hyaluronidase presented even higher values of absorbance, showing that 75 IU of this hyaluronidase would be sufficient to degrade 0,1ml of HA (25mg/ml). Ideally, conducting additional measurements over time would allow for a more comprehensive analysis of the plateau phase in the results and provide more conclusive insights into the complete degradation of HA. Furthermore, it would be valuable to conduct more *in vivo* and clinical studies to investigate the action and applications of recombinant hyaluronidase.

Sall *et al*. served as a reference to this experiment, where they examined the degradation of 11 different HA fillers by bovine hyaluronidase. In their study, they observed absorbance values of 0,10-0,20 nm within 2h, a range that we could only achieve between 6 and 24 hours of incubation. [(50)] However, they used a concentration of 6080 IU/ml, which is approximately 8 times higher than the concentration used in our study, which can explain the faster degradation they observed. Although fast degradation of HA fillers may be desired in clinical situations, such high concentrations are generally not recommended. In this regard, our study could be a better option when extrapolated to *in vivo* clinical scenarios. However, ideally, our study would have tested different concentrations of various hyaluronidases to establish a Michaelis-Menten kinetics and determine the optimal dose of hyaluronidase for degrading a specific quantity of HA filler. Due to limited available materials in our study, further investigation is needed in this area, to determine the ideal hyaluronidase dosage.

Kim *et al*. examined the optimal timing for reinjection of HA fillers after treatment with hyaluronidase *in vivo*. They used 0,2ml of a HA filler with a concentration of 20mg/ml and 600 IU of hyaluronidase and concluded that after 3h of hyaluronidase injection, the reinjected filler nearly restored the shape and volume observed in the control group that received only HA injection. After 6h, there was no significant difference between this and the group injected solely with HA. [(51)] It is important to note that they used 600 IU of hyaluronidase, 300 IU for each 0,1ml of HA filler with a concentration of 20mg/ml. Once again, this hyaluronidase concentration is higher than the recommended dosage found in the literature. In our study, we used 75 IU of hyaluronidase per 0,1ml of HA filler (25mg/ml), a lower hyaluronidase concentration in relation to a slightly higher HA concentration. This difference in concentrations may account for the observed lower enzymatic activity and slower degradation

of HA, since in our study, the plateau was only achieved at approximately 24h. This aspect should be considered when extrapolating the findings to clinical applications, as a higher dose of hyaluronidase is more likely to cause a complication. [45]

While our study provides information about the degradation of different HA fillers using two different hyaluronidases, this is an *in vitro* study. Although we attempted to replicate conditions found in the human body, such as maintaining the appropriate temperature (37ºC) and pH (7.4) of the reaction, there are still factors to consider when extrapolating the results for an *in vivo* situation, such as the natural clearance of both HA and hyaluronidase that will occur when injected. Ideally, an *in vivo* study should be conducted in human tissues to better understand the applicability of these findings in daily clinical cases. Given the limited availability of materials, we suggest that future studies examine the degradation of fillers more frequently, ideally on an hourly basis, and extend the observation time to properly understand the point at which hyaluronidase completely degrades all hyaluronic acid.

6. Conclusion

In recent years, there has been a global increase in the use of HA fillers, leading to a growing demand for hyaluronidase, an antidote used in case of complications. However, despite the rising popularity of both HA fillers and hyaluronidase, there are still gaps in the existing literature about this theme.

The selection of hyaluronidase for clinical practice depends on the desired outcome and the specific characteristics of the HA filler being used. Different formulations of hyaluronidase may exhibit variations in enzymatic activity which affects its efficacy in degrading hyaluronic acid.

Our study aimed to compare the effectiveness of animal and recombinant hyaluronidases in degrading two different injectable HA fillers.

Comparing the four groups of study, the combination of ART FILLER® Volume with recombinant hyaluronidase demonstrated higher levels of degradation, consistently outperforming the other groups in most measurements over time.

When comparing the two HA fillers, no statistically significant differences were found, except at 1h, when animal hyaluronidase exhibited greater effectiveness in degrading ART FILLER® Universal compared to ART FILLER® Volume, making this hyaluronidase a suitable option for treating urgent complications associated with ART FILLER® Universal.

In terms of hyaluronidases, recombinant hyaluronidase appeared to be the most effective. However, the significantly higher degradation values compared to animal hyaluronidase were only observed when paired with ART FILLER® Volume and after 6h of incubation. Therefore, in clinical practice, when treating a complication associated with prior injection of ART FILLER® Volume, recombinant hyaluronidase should be the preferred enzyme.

Although our study provided valuable insights, it is important to acknowledge its limitations. The restricted availability of materials limited our ability to test multiple concentrations of hyaluronidase and work with larger sample sizes. Including multiple concentrations of hyaluronidase would have allowed a more comprehensive evaluation of enzymatic activity and effectiveness, facilitating the determination of the optimal dosage. Further investigations should address these limitations to enhance our understanding of these treatments.

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