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Faculdade de Medicina Dentária

Transarterial hyaluronidase activity in injectable hyaluronic acid - preliminary in vitro study

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Dissertação
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ABSTRACT

Aim: This pilot study intend to evaluate the knowledge about transarterial effectiveness of hyaluronidase activity and validate a new *in vitro* protocol to research this activity on Hyaluronic Acid (HA) injectable fillers.

Methods: Fresh pig skin samples were collected from a local slaughterhouse. Then the pig's fresh facial arteries were dissected. All of the 5 arteries were initially tied off with a 3.0 silk suture and then weighed in the electronic microbalance (Wi). After this the filler was injected into one artery and all groups were prepared and weighed (Wreagent). The artery number 1 (A1) corresponds to the silicone immersed in Hyaluronidase (HYAL), artery number 2 (A2) corresponds to the filler immersed in HYAL, the artery number three (A3) corresponds to the filler in saline solution, The artery number four (A4) is the empty artery in HYAL. Finally, the artery number five (A5) is the empty artery in saline solution. Then we proceeded to the sequential weight measures in the microbalance after their preparations at 20min, 30min, 60min, 90min, 6h and 24h (Wfinal).

Results: The A3, containing filler immersed in saline solution had a progressive increase in its weight. From all of the groups, only the artery with HA submersed in Hyal (A2) showed a decrease in its weight.

Conclusion: Within the limitations of this pilot study, and according to the results described in the literature, it is possible to conclude that this preliminary study allows to evaluate, in vitro, the HA intra-arterial degradation by HYAL in a pig's artery and it may represent a promising protocol regarding its future use to evaluate the efficacy of subcutaneous injection of HYAL in degrading injectable HA.

Keywords: Hyaluronic acid; Hyaluronidase; Transarterial activity; Artery

RESUMO

A busca pela beleza é universal e pode ser abordada a partir de diversas perspetivas, com a obtenção de vários resultados. O processo de envelhecimento facial é multifatorial, resultando de alterações em todas as camadas, nos tecidos moles e no esqueleto subjacente. Atualmente, o uso de fillers injetáveis é o segundo procedimento mais utilizado na harmonização facial. Nos dias de hoje, o filler injetável mais utilizado é o Ácido Hialurónico (AH), seguido do Hidroxiapatite de Cálcio (CaHA) e do Ácido Poli-L-Láctico (PLLA).

A capacidade do ácido hialurónico de ligar um grande número de moléculas de água aumenta significativamente a hidratação dos tecidos e a resistência destes a danos mecânicos. A sua popularidade no campo da medicina deve-se à sua ampla disponibilidade, à sua completa reabsorção (degradada pela hialuronidase) e à sua biocompatibilidade. Dado que o sinal primordial do envelhecimento da pele é a diminuição na concentração de AH na pele, a injeção de AH reversível é vista como o gold standard para o rejuvenescimento e/o aumento de tecidos, hidratação profunda da pele e remodelação facial.

Embora as injeções de preenchimento com AH tenham um perfil de segurança alto, podem ocorrer eventos adversos. Felizmente, a maioria dessas reações adversas é mínima e temporária; no entanto, em ocasiões raras, algumas delas podem ser extremamente significativos.

Problemas vasculares são os mais graves de todas as potenciais complicações e podem resultar em cegueira (por oclusão da artéria retiniana) e necrose da pele. Supõe-se que a injeção intravascular, que pode envolver dano vascular parcial ou completo, é a principal responsável pelos problemas vasculares. Esse problema pode resultar em efeitos de necrose locais e à distância.

Atualmente, não existe uma terapia estabelecida para os problemas vasculares relacionados ao AH. Algumas das terapias recomendadas incluem compressas quentes, pasta de nitroglicerina, terapia com oxigénio hiperbárico, massagem para remover o êmbolo do preenchimento e injeção de hialuronidase na área afetada. O componente principal do tratamento é o uso da hialuronidase.

A hialuronidase (HYAL) é uma enzima que hidrolisa tanto preenchimentos dérmicos de AH natural como com *crosslinking*, que resulta na formação de uma rede tridimensional, devido a uma modificação química utilizando agentes de crosslinking, e que melhora as caraterísticas viscoelásticas do AH e aumenta a sua resistência à degradação pela HYAL, à oxidação por radicais livres e tensões mecânicas. As hialuronidases são enzimas endo-N-acetil hexosaminidases que catalisam polímeros de AH em monossacarídeos, dividindo ligações glicosídicas. A HYAL é frequentemente utilizada fora das indicações aprovadas, especialmente na dermatologia estética, para tratar os efeitos indesejados dos preenchimentos de AH, como sobrecorreção ou implantação superficial de AH, reações granulomatosas a corpos estranhos e tratamento de necrose da pele. É usada na medicina estética tanto para procedimentos eletivos como para procedimentos de emergência.

As hialuronidases atualmente disponíveis para uso médico são de origem animal ou do tipo recombinante humano. Os produtos aprovados pela FDA incluem hialuronidase testicular bovina, hialuronidase testicular ovina e hialuronidase humana recombinante.

O comprometimento vascular após uma injeção de preenchimento de tecido mole é uma complicação importante e imediata que resulta quase sempre de uma injeção intravascular em uma artéria, causando um êmbolo que impede o fluxo sanguíneo. Clinicamente, é caraterizado pelo início súbito de dor após a injeção, coincidente com reticulação viva, formação de bolhas, necrose dérmica, demarcação e, posteriormente, desenvolvendo-se numa úlcera necrótica e cicatrizes tardias.

Quando se suspeita de uma oclusão vascular, é crucial interromper imediatamente a injeção. O objetivo é facilitar o fluxo sanguíneo para a área afetada. O uso da HYAL na dissolução de AH reticulado é altamente eficaz e tem vindo a mostrar a sua eficácia também a nível da prevenção da necrose tecidual. Para evitar a necrose da pele, a hialuronidase deve ser injetada ao longo do curso da artéria envolvida, para permitir a restauração do fluxo sanguíneo. A hialuronidase pode ser aplicada de maneira eficaz dentro das primeiras 4 horas, após verificar a complicação. A aplicação tardia de hialuronidases (mais de 24 horas após a injeção de AH) não se mostrou eficaz na prevenção da necrose da pele. No entanto, o seu uso pode reduzir o tamanho da área necrótica e melhorar o processo de cicatrização.

Um novo protocolo pulsado de alta dose foi estabelecido. O Collaborative Complications in Medical Aesthetics (CMAC) recomenda o uso de 1500 unidades por 1 mL, se disponível, como volume de reconstituição e a utilização de um modelo pulsado de alta dose. A CMAC estimou que a redosagem deveria ser feita em torno de 15 a 20 minutos, se necessário, após a dose inicial, após reavaliação.

O principal objetivo deste estudo foi fazer uma revisão da literatura atual sobre a eficácia da atividade transarterial da hialuronidase e tentar avaliar e validar um novo protocolo para investigar esta atividade com um filler de AH injetável.

Este estudo experimental preliminar in vitro foi conduzido na Faculdade de Medicina Dentária da Universidade de Lisboa para avaliar e validar um protocolo de investigação sobre a atividade transarterial da hialuronidase na degradação do AH injetável ao longo do tempo.

Amostras frescas de pele da face do porco foram recolhidas de um matadouro local. Em seguida, foram dissecadas as artérias faciais frescas do porco. Todas as 5 artérias foram inicialmente suturadas nas extremidades com uma sutura de seda 3.0 e depois pesadas num microbalança eletrónica (Wi). De seguida, foram injetadas no interior de uma artéria 0,3 ml de filler (Artéria 2) e as restantes 4 foram utilizadas como grupo controlo. De seguida, as cinco artérias foram novamente pesadas com os respetivos reagentes já no seu interior (Wreagent). Nos grupos de controlo positivos, foi injetado silicone light na artéria e inserido num tubo Eppendorf com 1 ml de solução de hialuronidase (Artéria 1) enquanto na outra artéria também foi injetado 0,3 ml do mesmo filler e esta foi depois inserida num tubo Eppendorf com 1 ml de solução salina (Artéria 3). Nos grupos de controlo negativos, nenhum preenchimento foi injetado nas artérias; a artéria 4 foi inserida num tubo Eppendorf com 1 ml da mesma solução de hialuronidase, e a artéria 5 foi colocada num tubo Eppendorf com 1 ml de solução salina. Em seguida, procedemos às medidas sequenciais de peso na microbalança após as suas preparações aos 20 minutos, 30 minutos, 60 minutos, 90 minutos, 6 horas e 24 horas (Wfinal).

De acordo com os resultados obtidos para o peso ao longo do tempo, é possível observar que aos 20 minutos houve um aumento de peso em todas as artérias, à medida que se tornavam mais hidratadas após serem imersas em diferentes substratos. No entanto, o aumento de peso, neste momento, nas artérias contendo filler e foi significativamente

maior devido à capacidade do filler de absorver água, ou seja, devido ao seu poder hidrofílico. Quando colocado em solução salina, observámos esse comportamento hidrofílico do preenchimento, pois houve um aumento progressivo de peso. Pelo contrário, na artéria com preenchimento imersa em hialuronidase, observa-se uma diminuição de peso a partir de 20 minutos em diante, que se deve à atividade enzimática.

Em linha com os estudos existentes, teve-se como objetivo a criação de um protocolo no qual se pudessem obter resultados quantitativos sobre a atividade transarterial da HYAL usando artérias.

O facto deste ser um estudo in vitro, com parâmetros controlados, não mimetiza o que acontece em cenários clínicos, como a desativação e migração da HYAL, profundidade do preenchimento, localização e volume. No presente estudo, o AH está sempre em contacto com esta enzima, o que pode contribuir para uma degradação mais rápida e eficaz do preenchimento.

Dentro das limitações deste trabalho enquanto estudo piloto e com as suas concordâncias com estudos passados, é possível concluir que este pode representar um protocolo promissor no que diz respeito ao seu uso futuro para avaliar a eficácia da injeção subcutânea de HYAL na degradação de AH injetável em casos de complicações vasculares e tentar estabelecer novos protocolos ou sustentar os protocolos que já foram sugeridos, de forma a tentar controlar estes eventos vasculares.

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CaHA – Calcium Hydroxyapatite

CMAC – Complications in Medical Aesthetics Collaborative

DMEM – Dulbecco's Modified Eagle Medium

FDA – Food and Drug Administration

HA – Hyaluronic Acid

HYAL – Hyaluronidase

PLLA – Poly-L-Lactic Acid

I. INTRODUCTION

The pursuit of beauty has been universal and it can be approached from a variety of perspectives, with varying outcomes. Our faces are composed of four major structural components: skin, fat, muscle, and bone.[1] The facial aging process is multifactorial, resulting from changes in all layers, the soft tissue and the underlying skeleton.[2] Many visible signs of aging are caused by combined effects of gravity, bone resorption and decreasing elasticity in these structures as we age resulting in wrinkles, loose skin and other recognizable indications of aging process.[1, 3]

Currently, the use of injectable fillers is the second facial aesthetic leading procedure.[4] This substantial growth is understandable given their simple administration, affordability, notable aesthetic improvements, and relatively low occurrence of complications.[5]

1.1 Dermal Fillers

Injectable fillers were introduced in medicine as a method for rejuvenating the aging face and repairing facial deformities.[1] They provide excellent nonsurgical solutions for anti-aging and enhancing facial features, targeting particular areas of the face to reduce lines, wrinkles, hollowing, and enhance features. Fillers' main indications are the filling of rhytides and folds as well as correction of soft tissue that has been lost due to disease or aging.[6] More and more, they can be used for a wide range of applications, including wrinkles (from fine to deep), facial deformities, sunken scars, HIV-related lipoatrophy, folds, volume replacement and enhancement, cheek and chin augmentation, tear trough correction, nose reshaping, midfacial volumization, lip enhancement, hand rejuvenation, and correcting facial asymmetry.[1, 6] The FDA-approved indications for dermal fillers are treating facial wrinkles and folds in the mid to deep dermis, addressing perioral rhytids, adding volume to the dorsum of the hands, augmenting lips, correcting contour deficiencies and improving the appearance of acne scars.[1, 5]

We cannot exclude the fact that the fillers are still foreign bodies in our system.[1] The number of complications will most likely rise along with the number of indications and performed procedures.[6] Therefore, the perfect dermal filler should be safe, affordable, hypoallergenic, simple to distribute, easy to store, quickly injectable and painless; should also not necessitate allergy testing; cause any downtime on the patient; and have no risk

of complications. Additionally, its results should be long-lasting, consistent, predictable, and simple to remove if necessary. They should also feel natural on the skin.[1, 5, 7]

The FDA has approved a number of filler materials, each with a different composition, injection profile and duration of effect. Currently, the most used injectable filler is Hyaluronic Acid (HA), followed by Calcium Hydroxyapatite (CaHA) and Poly-L-Lactic Acid (PLLA).[1, 5]

1.1.1 Fillers Classification

Dermal fillers can be classified in respect to their material properties, biodegradability, and duration of effect.

1.1.1.1 Material Properties

Fillers can be categorized as autologous, heterologous (or biological fillers), and alloplastic (or synthetic material) based on the material properties.[1, 5] Autologous fillers are obtained from the same individual and its own tissue including dermis, fascia, cartilage and fat (eg: autologous fibroblasts).[1, 5] Heterologous or biological fillers are derived from different species (eg: Hyaluronic acid and collagen).[5] Alloplastic or synthetic material, are manufactured from non-biological materials like metal, ceramic, or plastic.[5]

1.1.1.2 Biodegradability and duration of effect

Dermal fillers can be categorized in a number of ways, but for a better understanding of its complications, it is most helpful to distinguish between biodegradable (moderate and long-lasting) and non-biodegradable or permanent fillers.[5, 6] Biodegradable fillers are rapidly reabsorbed by the body, so they provide transitory and short-lived (3-6 months)[8] cosmetic effects such as hyaluronic acid (HA) and collagen.[1, 6, 8] Long duration or long lasting biodegradable fillers have a longer-lasting duration of effect (6-24 months)[8] and stimulate the body to synthesize its own collagen [5] such as calcium hydroxyapatite and L-polylactic acid (PLLA).[8, 9] Non-biodegradable or permanent (> 24 months)[8] stimulates a fibroblastic deposition of collagen around the nonabsorbable microspheres such as polymethyl methacrylate (PMMA)[1, 5, 6], which is the only permanent filler

approved by the FDA.[1, 5, 7] The main concerns with the use of permanent fillers are the potential late-onset reactions or displacement of the material as facial structures alter with the aging process.[7]

In both Europe and the USA, HA derivatives are the most often used biodegradable fillers. Depending on the source and degree of cross-linking, concentration, and particle size of each product, effects typically last 6 to 18 months.[6]

2. Hyaluronic Acid

The ability of hyaluronic acid to bind a large number of water molecules significantly raises tissue hydration and increases the tissues' resistance to mechanical damage.[8, 10] Its popularity in the field of medicine is due to its widespread availability, complete resorbability (degraded by hyaluronidase), and biocompatibility.[8, 10, 11, 12] It's interesting to note that HA has the same structure in bacteria and vertebrates.[10] It is a substance that is excellent for soft tissue augmentation and face rejuvenation since it is structurally constant across species.[11]

Almost every tissue in all vertebrates contains HA.[13, 14] Mammals' connective tissues, including cartilage, synovial fluid, skin, rooster combs, umbilical cords, and vitreous humor, have large concentrations of HA.[10, 14, 15] The skin has the largest concentration of HA, making up nearly half of the overall amount.[8, 10, 13] The normal turnover rate of HA is 5g/day, its concentration in the skin accounts for 50% of its total body volume, and its half-life is 24 to 48 hours.[8, 16, 17] Given that the fundamental sign of skin aging is thought to be a decrease in the skin's HA concentration, injecting reversible HA is seen as the gold standard for tissue augmentation, deep skin hydration, and facial recontouring.[18]

2.1 Structure

Hyaluronic acid is composed of repeating units of two monosaccharides, namely D-glucuronic acid and N-acetyl-D-glucosamine.[5, 10, 11, 19, 20] This unique composition enables it to bond water molecules and assist the skin in improving its hydration levels and increasing its turgor. The monosaccharides are linked together via alternating beta-1,4 and beta-1,3 glycosidic bonds.[5, 10, 11, 13, 20, 21] It is naturally synthesized by a class of

integral membrane proteins called hyaluronic acid or hyaluronan synthases and degraded by a family of enzymes called hyaluronidases.[15] The basic unite of HA is the same regardless of its origin (eg: animal or bacterial) but its length differs.[19]

2.2 Properties

Various HA fillers differ in characteristics such as concentration, degree of cross-linking and viscosity.[3] Hyaluronic acid can be modified to increase its effectiveness by cross-linking its dimers, controlling the degree and way of chain cross-linking, the homogeneity and size of its particles and its concentration. These aforementioned elements play a significant role in determining the duration and efficacy of HA.[5, 13, 18] The longevity of injectable HA fillers is increased by a high HA concentration, higher HA particle size, and increased cross-links.[22]

2.2.1 Cross Linking

Stability, durability, and viscoelastic properties of the HA are all enhanced by synthetic cross-linking.[17] Unlike the indigenous HA polymers found in the human body, the majority of HA-based dermal fillers are made as crosslinked high-molecular weight HA polymers through chemical modification utilizing crosslinking agents.[11]

As a result of the crosslinking of HA polymers, a three-dimensional network is formed, which enhances the viscoelastic characteristics of HA fillers and increases their resistance to quick deterioration brought on by repeated exposure to endogenous hyaluronidase, free radical oxidation, and mechanical stresses.[11, 23]

When the degree of cross-linking and concentration of HA are increased, it results in higher viscosity and elasticity, along with higher resistance to degradation by native hyaluronidase (HYAL).[5, 11, 17, 19]

It should be noted that while more concentrated products with higher levels of cross-linking have effects that last longer, they also cause the body to become more reactive, increasing the risk of inflammation and granuloma development.[6]

2.2.2 Hydrophilic nature

Due to the hydrophilic nature of HA, products with higher concentration and/or larger particles tend to absorb more water. This will cause an increasing swelling of the tissue after injection.[5, 6]

2.2.3 Microsphere size

Another significant element that influences the expected outcomes is the size of the microspheres in the HA. HA can either be monophasic, with uniform microspheres, or biphasic, with a variety of microsphere diameters. Monophasic HA is regarded as the ideal solution.[5, 6] Since monophasic fillers are less exposed to HYAL than polyphasic fillers, they are less soluble in HYAL.[11, 24]

2.2.4 Hardness G'

Another element that impacts HA's efficacy is the hardness, measured by G'. The utility of various HA products for specific applications can vary depending on the degree of hardness in each product. Higher G' value products should typically be injected deeper into the tissue and is used in facial reconstruction and volumization.[5, 6]

2.3 Action mechanism

A vital part of the extracellular matrix found in the dermis is hyaluronic acid.[5, 25] It helps in the development of a resilient, gel-like ground material that can withstand compressive stresses.[5, 22, 26] Due to its water-binding properties, hyaluronic acid can form a highly viscous, hydrated polymer that maintains its volume by binding additional water molecules as it degrades.[22, 25] Due to the carboxyl groups in the molecule, HA is negatively charged and highly hydrophilic, HA matrix may retain up to 1000 times its weight in water.[25, 27] The turgor, or swelling pressure, that is produced when water is absorbed into the HA matrix enables the HA complex to resist those compressive stresses.[5]

2.4 Dermal fillers complications

Dermal fillers exist in a variety of forms, each with its own properties, potential risks and injection demands.[28] Although HA filler injections have a very favorable safety profile, adverse events can occur.[12] Fortunately, most of these adverse reactions are minimal and

temporary; but, on rare occasions, some of these adverse events might be extremely significant.[5] The common adverse events include local erythema, tenderness and swelling, all of which are related to the injection procedure.[29] In the case of other adverse events such as volume overcorrection and filler misplacement, the therapeutic effects of HYAL injections have been reported.[4, 29] It is important to note that with adequate preparation and execution, the majority of issues can be avoided.[5, 28]

2.4.1 Classification of dermal filler complications

Table 1 – Soft-tissue filler complications classification by Onset of adverse events.[4, 30, 31]

Adverse events	**Signs and symptoms**	
	Early events (Up to one week after treatment)	**Delayed events (Weeks to years after post-treatment)**
Injection site reactions	Erythema, Edema, Pain/tenderness, Bruising, Itching	Erythema, Edema, Pain/tenderness, Nodule/abcess, Systemic responses Biofilm
Infection	Erythema, Edema, Pain/tenderness, Acne papule formation, Nodule/abcess Herpes outbreak	Biofilm, Herpes outbreak, Foreign body granuloma

Hypersensitivity	Erythema, Edema, Pain/tenderness, Non-fluctuant nodules	Migration of filler material
Technical/placement errors	Bumps/Lumps, Asymmetries, Contour irregularities, Compromised muscle function, Dysesthesias, Paresthesias, Anesthesia	Immune reactions, Compromised muscle function, Dysesthesias, Paresthesias
Skin discoloration	Redness, Whiteness, Hyperpigmentation	Persistent discoloration Persistent scarring
Vascular compromise	Blurred vision, Loss of vision, Pain, Blanching	Tissue necrosis

The use of injectable HA fillers for cutaneous augmentation has considerably grown lately.[31] But when undesirable effects occurs can result in patient dissatisfaction, like unintended and excessive augmentations or the Tyndall effect (the injection of HA filler is too close to the superficial dermis or epidermis and can lead to a "bluish" skin discoloration that may be often confused with a bruise). So the reversibility of this process has gained importance and HYAL is the initial approach to treatment.[32, 33]

Vascular problems are the most serious of all potential complications and can result in blindness (when it causes retinal artery occlusion) and skin necrosis.[12, 30] It has been proposed that intravascular injection, that may involve partial or complete vascular damage, is the main responsible for vascular problems. But it may also result from

vascular compression caused by product excess. This issue could result in both local and distant necrose effects.[4, 11, 12, 34, 35, 36] Some anatomical areas, such as glabella, alar base, nose and temple are known to be associated with higher risks of vascular complications.[21, 30, 37] Currently, there is no established therapy for HA vascular problems. Some of the recommended therapies include warm compression, nitroglycerine paste, hyperbaric oxygen therapy, massage to remove the filler embolus, and injection of hyaluronidase into the afflicted area. The main component of the treatment is hyaluronidase.[4, 12, 34, 37]

3. Hyaluronidase

Hyaluronidase is an enzyme that hydrolizes both natural and crosslinked HA dermal fillers.[22, 32, 38, 39] HYAL is also thought to play important roles in many natural biochemical processes and has proven to be useful in clinical medicine.[32] Hyaluronidases are endo-N-acetyl hexosaminidases enzymes that catalyze HA polymers into monosaccharides by cleaving glycosidic bonds.[11, 24] They have numerous effects on tissues, including lowering HA's high viscosity, decreasing its lubrification ability, and serving as a "spreading factor" to aid in the diffusion of a number of subcutaneously injected compounds.[23]

The current U.S. Food and Drug Administration - approved indications recommend the use of HYAL for hypodermoclysis, to increase the subcutaneous absorption of beneficial drugs, disperse harmful injected drugs, treat extravasation injury, and improve the absorption of radiopaque agents during subcutaneous urography.[24, 40] Hyaluronidase is often used off-label for application in aesthetic dermatology, particularly for treatment of the undesirable effects of HA fillers, like overcorrection or superficial HA implantation, granulomatous foreign body reactions and treating skin necrosis.[23, 24, 38, 40, 41, 42, 43, 44] It is used within aesthetic medicine for both elective and emergency procedures.[45]

In humans, there are six different hyaluronidases that come from hyaluronidase-like genes HYAL1, HYAL2, HYAL3, HYAL4, PH20/SPAM1, and the pseudogene HYALP1.[11, 24, 46] The two main acid-active hyaluronidases found in most tissues are HYAL-1 and HYAL-2 that degrade HA by hydrolyzing the β-1,4 glycosidic linkage

between N-acetyl-glucosamine and D-glucuronic acid.[11, 39] HYAL-1 acts as a major hyaluronidase in plasma and is activated at an acidic pH. HYAL-2 exerts weaker enzymatic activity than hyaluronidase 1 and only breaks down high molecular weight hyaluronic acid.[24]

There is a classification for the three distinct groups of HYAL: (1) mammalian, (2) bacterial, and (3) from leeches, crustaceans, and some parasites.[23, 32] First, mammalian hyaluronidases are endo-β-N-acetylhexosaminidases that break down β-1,4 glycosidic linkages to form tetrasaccharides. Second, leech/hook-worm hyaluronidases are endo-β-D-glucuronidases that break down β-1,3 glycosidic bonds to form pentasaccharides and hex- asaccharides. Finally, microbial hyaluronidases are classified as hyaluronate lyases. Unlike other hyaluronidases, they do not catalyze hydrolysis reactions; instead, they produce unsaturated disaccharides through a β-elimination reaction at β-1,4 glycosidic linkages.[23, 24, 39, 46]

Hyaluronidases can also be classified into two types according to the pH at which they are most active. Acid-active hyaluronidases are activated at a pH of 3 to 4. Neutral-active hyaluronidases are activated at a pH of 5 to 8.[23, 24]

Hyaluronidases currently available for medical use are of either animal origin or human recombinant type. Products approved by the FDA include bovine testicular hyaluronidase, ovine testicular hyaluronidase, and recombinant human hyaluronidase.[44]

Allergic reactions are the only complications reported after Hyaluronidase injection.[23] These allergic reactions are well documented, but uncommon (0,05%).[38, 44] When treating non-emergency complications of HA dermal fillers, such as overcorrection, superficial implantation or inflamatory reactions, skin pretesting is considered optional. If it is an urgent case, such as vascular occlusions, no pretest is performed.[38, 44] In these cases, epinephrine should be available.[38, 44] The skin test is performed with 3U of Hyaluronidase.[24]

3.1 How to use Hyaluronidase to correct vascular complications

Vascular compromise after a soft tissue filler injection is a major, immediate complication that is almost always the result of intravascular injection into an artery, causing an embolism that impedes blood flow.[31]

The two primary diagnostic early symptoms of vascular occlusion are pain and changes in skin color.[21, 31, 47] In the course, pain may increase and reddish to bluish discolorations may occur which may then progress to tissue necrosis.[21] It is clinically characterized by the sudden onset of pain after injection, concurrent with lived reticular, blistering, dermal necrosis, demarcation, and subsequently developing into a necrotic ulcer and late scarring.[33, 34, 38]

When vascular occlusion is suspected, it is crucial that the injection is stopped immediately. The objective is to facilitate blood flow into the affected area.[30, 31, 37]

The use of HYAL in dissolving cross-linked HA is highly effective and has been shown to prevent tissue necrosis.[34, 37] To avoid skin necrosis, hyaluronidase should be injected along the course of the involved artery, to allow blood flow restoration.[21, 35, 38, 40, 47]

The injected HA can lead to cutaneous ischemia through compression or embolization of the subdermal plexus.[33, 47] Within a few hours, the skin may discolor, while necrosis and ulcers may become visible within 24 hours.[23] Hyaluronidase can be effectively applied within the first 4 hours.[21, 23 44, 47] Late application of hyaluronidases (more than 24 hours after HA injection) has not proven effective in preventing skin necrosis.[21, 23, 44, 47] However, their usage may reduce the size of the necrotic area and enhance the healing process. [23]

A new high-dose pulsed protocol has been established. The Complications in Medical Aesthetics Collaborative (CMAC) recommends using 1500 units per 1mL, if available, as the reconstitution volume and employing a high-dose pulsed model of dosing.[33, 37, 48]

CMAC estimated that the re-dosing should be around of 15 to 20 minutes, if required, after the initial dose following reassessment.[45] CMAC also advises the co-administration of Hyaluronidase with lidocaine without adrenaline.[45]

With appropriate treatment with the new High Dose Pulsed HYAL protocol, it is typically seen a complete reversal of all the signs of ischemia and complete return to normal within 3 days of the event, with no signs of secondary problems, instead of 6 weeks.[33]

II. OBJECTIVES

The main objective of this study was to do a review of the actual literature about the transarterial activity effectiveness of hyaluronidase and to try to evaluate and validate a new protocol to investigate this activity with one injectable HA filler.

III. MATERIALS AND METHODS

This preliminary *in vitro* experimental study was conducted in the Faculty of Dental Medicine of the University of Lisbon to evaluate and validate an investigation protocol about the transarterial activity of hyaluronidase in injectable HA degradation through time.

To perform this study, we dissected pigs' fresh facial arteries with approximately 2 cm each. To initially go unconscious, the pigs from a nearby slaughterhouse (Sicasal, Mafra, Portugal) were put inside a 98% carbon dioxide chamber. The carotid artery was then cut, which resulted in their death. Immediately upon the death of the pigs, two symmetrical skin samples were taken using a scalpel with a 15-blade, dissecting forceps, detacher, and metal ruler. As soon as possible, the pieces were put in containers with Dulbecco's Modified Eagle Medium (DMEM) (Biowhittaker®, Walkersville, Maryland, United States), in a sufficient volume for each piece to be immersed, at 37° C, in order to maintain the tissue vitality.

During the transportation process to the laboratory the arteries were conserved in a DMEM solution[49] and then put at 4ºC to prevent the cellular decomposition.

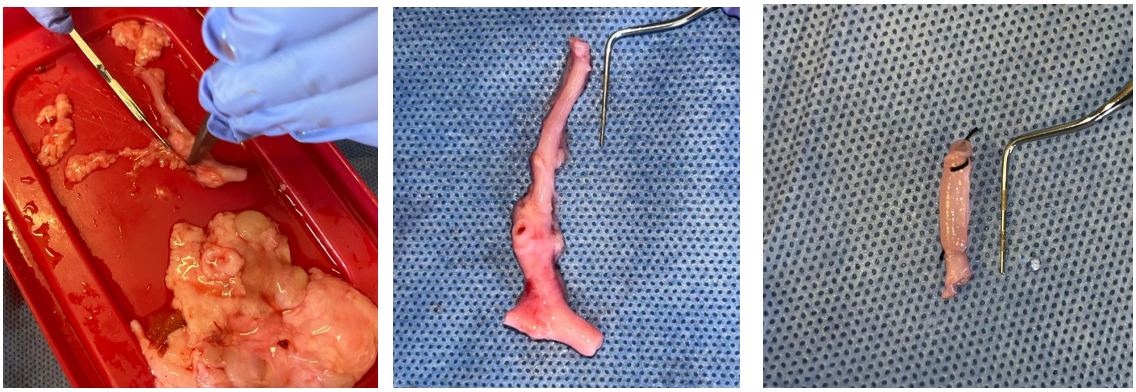


Figure 1 - Artery dissection and preparation.

All of the 5 arteries were initially tied off with a 3.0 silk suture and then weighed in the electronic microbalance (Wi).

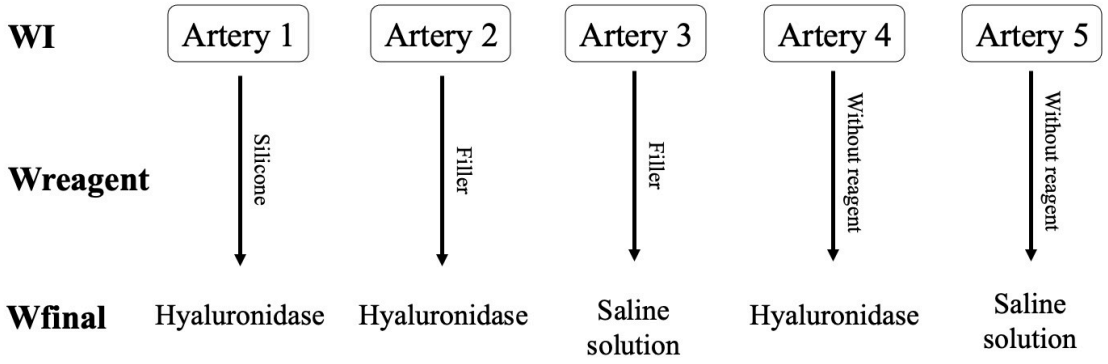


Figure 2 – Sample scheme.

After this step we prepared study group with the injection of 0,3ml of filler, enough to create a bolus in the artery (Artery 2). The selected filler was Art Filler Universal (Filmed®, France) 25mg/ml. After the artery preparation and its measure (Wreagent) it was inserted on an Eppendorf with 1ml of animal BCN hyaluronidase solution (Institute BCN®, Barcelona, Spain). We chose to follow the manufacturer's recommendations, which resulted in a concentration of 375 IU of HYAL/mL. This solution was obtained by diluting the liofized powder of 1500U in 4ml of saline solution in order to perform a final concentration of 375UI/ml.

Simultaneously, we prepared the control groups. In the negative control groups, no filler was injected in the arteries, after this the artery 4 was measured and inserted on an Eppendorf with 1ml of the same hyaluronidase solution and the artery 5 was also measured and put on an Eppendorf with 1ml of saline solution.

In the positive control groups, we injected light silicone in the artery, measured it and inserted on an Eppendorf with 1ml of hyaluronidase solution (Artery 1) and the other artery was also injected 0,3ml of the same filler and posteriorly measured and inserted on an Eppendorf with 1ml of the saline solution (Artery 3).

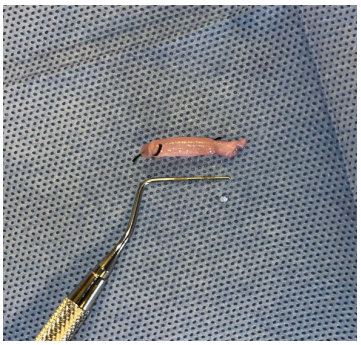


Figure 3 – Artery containing filler.

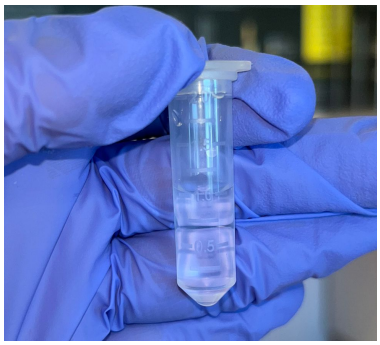


Figure 3 – Eppendorf with 1ml of Hyaluronidase (375UI/ml).

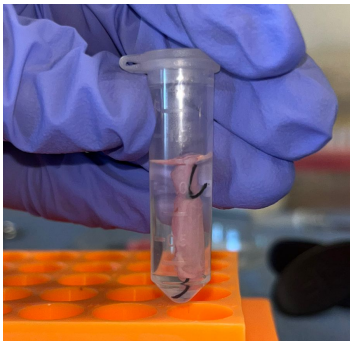


Figure 5 – Artery with filler submersed in 1ml of hyaluronidase.

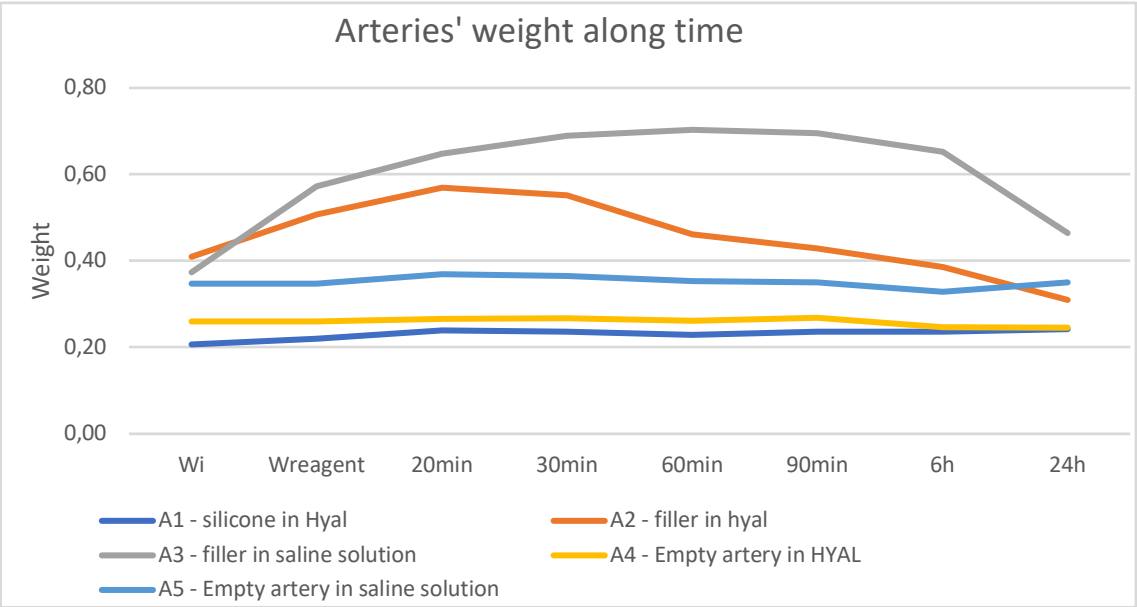
After the submersion of the different arteries in each substrate, every sample went to the incubator at 37ºC [12] and then we proceeded to the sequential weight measures in the microbalance after their preparations at 20min, 30min, 60min, 90min, 6h and 24h (Wfinal). Between each measure the preparations were again put in the incubator. Before each measure, the arteries were dried with paper tissues for 3 seconds to take off the excess oh hyaluronidase and saline solution.

IV. RESULTS

Table 2 – Weight measures along time.

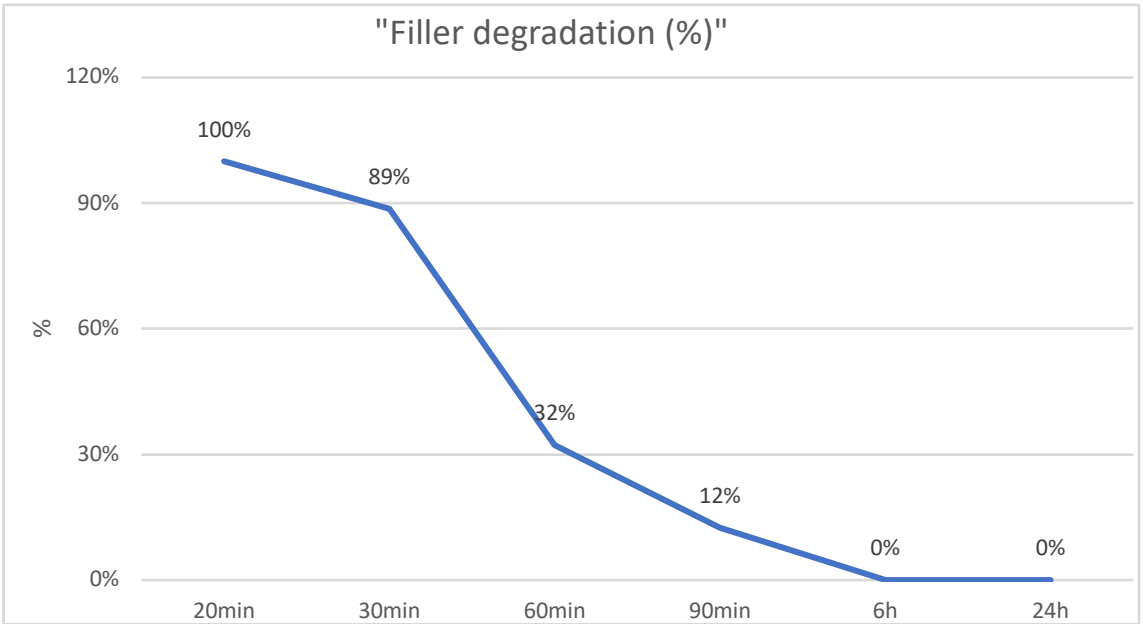
Weight along time					
Groups	A1 - Silicone in HYAL	A2 - Filler in HYAL	A3 - Filler in saline solution	A4 – Empty artery in HYAL	A5 – Empty artery in saline solution
Wi	0,2067	0,4089	0,3735	0,2599	0,3467
W with reagent	0,2193	0,5079	0,5723	0,2599	0,3467
20min	0,2386	0,5693	0,6483	0,2660	0,3692
30min	0,2364	0,5510	0,6888	0,2672	0,3657
60min	0,2292	0,4607	0,7031	0,2620	0,3535
90min	0,2368	0,4289	0,6949	0,2684	0,3509
6h	0,2358	0,3851	0,6530	0,2469	0,3285
24h	0,2421	0,3097	0,4643	0,2455	0,3502

This table shows all the measures that were done over time. According to the results obtained for weight over time, it is possible to observe that at 20 minutes there was an increase in weight in all arteries, as they become more hydrated after being immersed in different substrates. However, the weight increase at this time in the arteries containing filler is significantly higher due to the filler's ability to absorb water, that is, due to its hydrophilic power. When placed in saline solution, we observe this hydrophilic behavior of the filler, as there is a progressive increase in weight. On the contrary, in the artery with filler immersed in hyaluronidase, a decrease in weight is observed from 20 minutes onwards. Both in the artery with filler immersed in hyaluronidase and in the artery without anything immersed in hyaluronidase, a decrease in weight towards its original value is observed between 90 minutes and 6 hours.



Graphic 1 – Arteries' weight along time.

This graphic translates the values of the arteries over time. The artery number one (A1) corresponds to the silicone immersed in HYAL and we can observe that there wasn't a great variety in its weight. The artery number two (A2) corresponds to the filler immersed in HYAL where we can observe an initial increase in its weight but also a decrease after 20min. The artery number three (A3) corresponds to the filler in saline solution and we observe an increase in its weight until 90min. The artery number four (A4) is the empty artery in HYAL and its weight was the same through time. Finally, the artery number five (A5) is the empty artery in saline solution and had the same behavior as the A4, its weight didn't change much. Being more visual, it is easier to verify that the greatest weight increase corresponds to the artery with filler in the saline solution (A3). Next, the greatest increase was observed in the artery with filler in the HYAL-containing medium, with this weight starting to decrease at 20 minutes. The arteries A1, A4 and A5 had a similar behavior as we can see that there was no significant variety in their weights.



Graphic 2 – Filler degradation (%).

This graphic shows the percentage of degraded filler over time, where it is possible to observe that at 90 minutes, it had been almost completely degraded. We also note that the highest percentage of degradation occurred between 30 minutes and 60 minutes.

V. DISCUSSION

The present study was a literature review for contextualizing the importance of more knowledge in this area and to do some research to preform also a preliminary in vitro study of singular importance, regarding the possibility of testing and evaluating parameters and action protocols in order to plan future studies in this same area.

The main objective of this study was to test the viability of a new technique that allows evaluating the transarterial capacity of the HYAL enzyme in degrading injectable hyaluronic acid.

Despite the safety profile of the HA based fillers' applications, side effects can still occur.[12, 34] By the possible utilization of the HYAL enzyme, there is now a safe and effective way of minimizing the adverse effects of HA fillers.[21, 47]
Since there is little literature regarding a specific protocol for addressing vascular obstruction and other complications arising from dermal fillers, more studies and research are needed to achieve a more elaborated action protocol.

DeLorenzi, in 2014, published the first study that came to prove that HYAL could go through an intact arterial wall. In that study, he has immersed fresh, human cadaver sourced facial artery segments filled with HA into a HYAL solution and demonstrated dissolution of the whole gel within 4h.[34] The main difference in these two studies lies in the fact that in his study only qualitative results could be obtained because it was observed under direct visualization with loupe magnification.[34] In our study quantitative results can be obtained with a larger sample.

Until the year of 2017, the HYAL injection site was controversial when in the presence of vascular compromise. In 2017, Whang *et al.* demonstrated that the subcutaneous injection of HYAL is more effective when treating obstructed vessels and degrading the HA filler when compared to the intra-arterial injection. This is due to the resistance caused by the filler in the artery which makes the enzyme diffuse to the open instead to the obstruction.[12] This fact has a great importance in this study given that this is the reason why we chose to try a new technique to evaluate the effectiveness of this process

that can allow us to take further conclusions about this theme and to try to understand which protocol should be established in cases of vascular compromise.

In 2020, Rauso *et al.* tried to reproduce two different clinic scenarios to mimetize the two mechanisms that may induce vascular compromise which are compression of the area around the vessel and obstruction/ embolization of the vessel by the filler material.[40] For simulating the embolization case, a vein specimen was filled with 0.25ml of a 20mg/ml HA cross-linked filler and soaked in a test tube containing 1ml of HYAL solution. After 2h of soaking the remaining quantities of HA were measured. In this embolization case, 0.15ml of HA filler was still detected.[40] The main difference between this study and our study as well as DeLorenzi's study may lie in the fact that Rauso *et al.* used a vein collected from a living patient. The fact that our study was performed with cadaver and non-human arteries is a limitation as there might have differences in the capability of HYAL to diffuse through a vessel wall between a living patients and cadavers.[40]

In 2017, DeLorenzi also proposed a new protocol for these vascular events that involves the administration of high doses of HYAL every hour until resolution.[33] Another fact that represents a limitation to our study and also supports this new protocol is the fact that this is an in vitro study, with controlled parameters that does not mimetize what happens in clinic scenarios such as HYAL deactivation and migration, filler depth, location and volume. In our study the HA is always in contact with this enzyme which may contribute to a faster and more effective degradation of the filler.

In line with the existing studies, we aimed to create a protocol in which we could obtain quantitative results regarding the transarterial activity of HYAL using arteries.

According to our results, we can see that the A3, containing filler immersed in saline solution had a progressive increase in its weight due to the hydrophilic activity of the filler, as well as its inherent expansion and level of cross-linking. The A2, with filler immersed in HYAL, had the second greatest weight increase, until 20min, also due to the hydrophilic activity of the filler. After 20min its weight starts decreasing as the enzymatic activity starts. We can also observe that at 90min this artery's weight containing filler was still superior to the artery weight itself which leads us to conclude that the filler was not all solved yet, but at 6h the weight of the artery containing filler is already inferior.

These results come to support Delorenzi's study once that he showed dissolution of the whole gel within 4h.[34] Also at 90min, we observed only 12% of remaining filler, while Rauso *et al.* observed 60% at 2h. These results are in contrast with the previous DeLorenzi's study and also with this study. Our results may also substant Delorenzi's protocol called "New high dose pulsed hyaluronidase protocol for vascular acid filler vascular events" (HDPH) given the fact that at one hour we still have 32% of remaining filler which means it could be necessary another HYAL injection at 1h.

In 2021, the Journal of Clinical and Aesthetic Dermatology also published a new Modified High Dose Protocol that suggests redosing of HYAL at 15 to 20 minutes, if required, after the first dose, due to HYAL's pharmacokinetic and its fast degradation once it reaches the systemic blood supply.[45] According to our results, at 20min we still have almost 100% of the filler which supports this new Modified High Dose Protocol, which means redosing at 15 to 20min until resolution given the HYAL degradation rate.

The main limitation of this study, we can mention its small sample size, which results from the economic costs associated with expensive materials such as the filler and the enzyme.

A difficulty encountered in conducting this study relates to the dissection of the arteries since we were looking for portions of the facial artery, and it was not always easy to identify it as we were managing pig's tissue with associated fat.

Another challenge to mention was the difficulty in injecting hyaluronic acid into the artery, as there was always leakage due to its high viscosity. As a consequence of the filler injection into the artery, it developed a perforation, which may have facilitated the passage of the enzyme and possibly contributed to the effective degradation of the filler and it may also have facilitated some filler extravasion from the artery. This also represents a limitation to this study.

One of the factors that most affected the results was the hydration of the arteries, and it is difficult to ensure that they were all at the same level of hydration during weighing. To mitigate this problem, before each weighing, all arteries were dried to try to eliminate excess substrate.

Considering the results observed when attempting to use this technique and comparing it to the studies that were made until today, it is possible to affirm that it can be extrapolated, and with a larger sample, we can draw important conclusions in this field. However, its reproducibility, effectiveness, and precision can only be determined with a more comprehensive sample. In the future, randomized in vitro studies should be carried out in order to evaluate the various enzymes and their ability to act through structures to try to standardize their clinical use.

VI. CONCLUSION

According to the literature review it is possible to conclude that new standardized protocols are needed to have a more efficient response to all of the possible fillers' complications and their consequences. Within the limitations of this study as a pilot study and with its concordations with the past studies it is possible to conclude that this preliminary study may represent a promising protocol regarding its future use to evaluate the efficacy of subcutaneous injection of HYAL in degrading injectable HA in cases of vascular complications and to try to establish new protocols or sustent the protocols that were already suggested such as the new Modified High Dose Protocol to try to control these vascular events.

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