Universidade de Lisboa

Faculdade de Medicina Dentária

EFFECTS OF INJECTABLE HYALURONIDASE ON INTRINSIC HYALURONIC ACID OF DERMAL TISSUE - PILOT STUDY

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Dissertação

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ABSTRACT

Aim: The aim of this pilot study is to evaluate the effects of the application of two hyaluronidase doses on dermal intrinsic hyaluronic acid, over time.

Methods: Fresh skin pig's nose were collected from a local slaughterhouse. Two study groups were evaluated after intradermal injection of 0,1 ml of 18,75 IU of HYAL (group A) and 37,5 IU of HYAL (group B). For comparative purposes, two control groups were employed: a group without injections as the negative control (group C), to assess possible postmortem changes, and a group subjected to 0,1 mL of saline solution injection as the positive control (group D). Skin biopsies were performed 1 hour and 6 hours after injections. An additional biopsy was performed at baseline without any injection. The samples were histologically processed and later submitted to Hematoxylin and Eosin and Alcian blue staining, for the study of morphometry and evidence of hyaluronic acid, respectively.

Results: Upon histological examination, the tissue architecture was found to be well-preserved in the initial control group, evidenced by the integrity of the epidermis, normal organization of dermis and conserved structure of subcutaneous layer. At 1 hour, the skin samples of the different groups also demonstrated preservation of tissue architecture. The intensity and distribution of Alcian blue staining was similar in all analyzed samples. The connective tissue was homogeneously stained, showing a light blue color.

Conclusion: The absence of observed histological changes through the uniformity of Alcian blue staining indicates the promising potential of injectable doses hyaluronidase between 18,75 IU and 37,5 IU as a safe intervention, for the time analyzed. This pilot study shows the suitability of the porcine model and the effectiveness of the methodology employed on it so it serves as a valuable starting point for future histological investigations.

Keywords: Hyaluronoglucosaminidase; Hyaluronic acid; Skin; Cosmetic Techniques; Histology; Models, Animals.

RESUMO

No processo de envelhecimento, a pele sofre alterações que resultam na redução de componentes essenciais, como o colagénio, elastina e ácido hialurónico. Essa diminuição contribui para as rugas, flacidez e perda de volume facial, características comuns do envelhecimento cutâneo. O ácido hialurónico desempenha um papel fundamental na hidratação, elasticidade e aparência jovial da pele. Na dermatologia estética, o ácido hialurónico é frequentemente utilizado como um preenchedor dérmico para restaurar a hidratação e volume facial perdido. No entanto, complicações podem ocorrer, desde resultados insatisfatórios, reações inflamatórias e infeções, formação de nódulos a obstrução vascular e necrose, ainda que raras. A hialuronidase é uma enzima importante no tratamento dessas complicações, dissolvendo o ácido hialurónico indesejado e revertendo os efeitos adversos.

A hialuronidase pode ser classificada em três tipos de acordo com seu mecanismo de ação: mamífera, de sanguessuga/ancilostomídeo e microbiana. As duas primeiras atuam por quebra das ligações glicosídicas no ácido hialurónico e noutros componentes, enquanto a hialuronidase microbiana atua por eliminação, resultando em dissacarídeos insaturados. As formulações de hialuronidase podem ser obtidas de fontes bovina, ovina ou bacteriana. Atualmente existe uma hialuronidase recombinante humana, que é considerada a mais segura e menos imunogénica. No tratamento de complicações relacionadas com o preenchimento com ácido hialurónico, a hialuronidase pode ser usada tanto em situações de emergência, das quais se incluem as complicações vasculares, como em situações eletivas, para corrigir resultados indesejados. As doses de hialuronidase variam dependendo da situação, mas geralmente são administradas entre 3 a 75 unidades internacionais, sendo recomendado avaliar os resultados após 48 horas e repetir o tratamento conforme necessário. É importante massajar a área após a aplicação da hialuronidase e aguardar pelo menos 3 horas antes de reaplicar o ácido hialurónico.

Apesar de muitos estudos já terem descrito a eficácia dos produtos de hialuronidase na resolução dos efeitos indesejáveis dos preenchedores de ácido hialurónico, pouca atenção tem sido dada à sua segurança. Alguns estudos avaliaram essa questão especificamente quanto à hialuronidase recombinante humana, embora não exatamente na pele. Mas poucos se focaram em estudar o mesmo para as hialuronidases de origem animal, que ainda são as formulações mais comercializadas. Por esta razão, uma análise histológica dos efeitos de uma hialuronidase bovina do mercado é realizada no presente estudo. Esta foi realizada em tecido cutâneo após a morte num porco, uma vez que este modelo é amplamente reconhecido como uma boa

representação da pele humana pelas inúmeras semelhanças, e não ocorrerem alterações significativas na derme nas primeiras 18 horas após a morte, quando mantida a 37º C. O ácido hialurónico intrínseco foi observado através da coloração Azul alciano, que tem sido amplamente utilizada para evidenciá-lo.

O objetivo deste estudo piloto é avaliar os efeitos da aplicação de duas doses de hialuronidase no ácido hialurónico intrínseco dérmico, ao longo do tempo.

Um estudo experimental *ex-vivo* foi conduzido na Faculdade de Medicina Dentária da Universidade de Lisboa para avaliar os efeitos de diferentes doses de uma hialuronidase bovina injetada nos níveis de ácido hialurónico endógeno na pele de porcos ao longo de um período de 6 horas.

O desenho do estudo consistiu em quatro grupos diferentes: injeção de duas doses diferentes de hialuronidase: 18,75 UI (grupo A) e 37,5 UI (grupo B), um grupo de controlo negativo sem injeções (C) e injeção de solução salina como controlo positivo (D), para comparação. Cada grupo contém 2 tempos de biópsia diferentes: 1 hora (A1, B1, C1 e D1) e 6 horas (A2, B2, C2 e D2) após a injeção, exceto o controlo negativo que apresenta uma biópsia adicional ao momento inicial (C0).

Para obtenção das amostras de pele, porcos de um matadouro local foram sacrificados e foram coletadas amostras de pele simétricas com medidas de 5,5 x 10,5 cm cada, imediatamente após a morte. As amostras foram colocadas em recipientes com DMEM de forma a manter a viabilidade do tecido.

Antes das injeções, foram realizados na pele pontos de sutura de forma a marcar os locais de injeção e posteriormente da biópsia. As injeções foram realizadas usando hialuronidase BCN, disponível no mercado e solução salina. Duas concentrações diferentes de hialuronidase (375 UI/mL e 187,5 UI/mL) foram preparadas diluindo um frasco de 1500 UI em solução salina. Um Médico Dentista calibrado e com experiência em harmonização facial realizou todas as injeções, garantindo a adequada desinfecção da pele antes de cada injeção e massajando após. As injeções foram administradas perpendicularmente à pele, na derme, usando uma agulha 28G e seringa de 1 mL.

Após as injeções, biópsias de pele foram feitas no momento inicial, após 1 hora e após 6 horas usando um bisturi circular de 4,5 mm de diâmetro. As amostras foram imediatamente fixadas em formaldeído a 4% e enviadas para análise histológica.

O processamento histológico das amostras foi realizado por uma estudante sob supervisão de uma especialista em anatomia patológica da Faculdade de Medicina Dentária. As amostras foram submetidas a fixação, desidratação, diafanização, incorporação em parafina e seccionamento usando um micrótomo. As seções resultantes foram então submetidas a coloração com Hematoxilina e Eosina e coloração com Azul alciano. A coloração H&E foi realizada para avaliar a morfometria, enquanto a coloração com Azul alciano foi utilizada para destacar o ácido hialurónico. As lâminas coradas foram examinadas usando um microscópio ótico e analisadas por um estudante com a ajuda do especialista.

Após a injeção, não foram observadas diferenças macroscópicas. Histologicamente, foi observada preservação da arquitetura tecidual no momento inicial e em todas os grupos após 1 hora, com observação da integridade da epiderme com distribuição adequada das suas camadas, organização normal da derme, presença de estruturas anexas intactas e tecido subcutâneo com estrutura preservada. Infiltrados inflamatórios foram observados em todas as amostras histológicas examinadas, com intensidade e distribuição semelhantes. A intensidade da coloração de Azul alciano, usada para visualizar o ácido hialurónico, foi semelhante em todas as amostras analisadas, tanto no momento inicial quanto após 1 hora da injeção de diferentes concentrações de hialuronidase, solução salina (controlo positivo) e ausência de tratamento (controlo negativo). O tecido conjuntivo foi corado de forma homogénea, mostrando uma coloração azul clara, e os núcleos celulares apareceram corados de vermelho.

Os resultados deste estudo não mostraram diferenças na quantidade ou distribuição do ácido hialurónico entre o momento inicial e 1 hora após a aplicação da hialuronidase em concentrações até 37,5 UI. Esses resultados divergem de estudos anteriores que observaram uma redução dos níveis de ácido hialurónico com concentrações mais baixas que as utilizadas neste estudo. Isto pode ser atribuído a uma diminuída sensibilidade da técnica que pode não detetar variações subtis nos níveis de ácido hialurónico tecidular.

Podemos encontrar algumas limitações neste estudo, como o recurso a um modelo *ex vivo*, que implica ausência de circulação sistémica e afeta consequentemente fatores importantes, nomeadamente a *clearence* da hialuronidase, e a utilização de um método de avaliação qualitativo e inespecífico para o ácido hialurónico. Ainda assim, a similaridade da pele entre o humano e o porco permitiu prever de forma fidedigna as alterações morfológicas e celulares.

A ausência de alterações histológicas observadas pela uniformidade da coloração do Azul alciano indica o potencial promissor de doses injetáveis de hialuronidase entre 18,75 UI e 37,5

UI como intervenção segura, para o tempo analisado. Este estudo piloto mostra a adequação do modelo suíno e a eficácia da metodologia nele empregada, servindo como um ponto de partida para futuras investigações histológicas.

Palavras-chave: Hialuronidase; Ácido hialurónico; Pele; Técnicas de cosmética; Histologia; Modelos animais.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABREVIATURES

GAGs – Glycosaminoglycans

HA – Hyaluronic acid

% - Percentage

μm – Micrometer

ECM – Extracellular matrix

H&E – Hematoxylin & Eosin

^{o}C – Celsious degree

GlcNAc – N-Acetylglucosamine

GlcA- Glucuronic acid

Da – Dalton

Na – Sodium

μg - Microgram

g – Gram

CD44 – Clusters of differentiation 44

RHAMM – Hyaluronic acid-mediated motility receptor

MW – Molecular weight

mg – Miligrams

HYAL – Hyaluronidase

ROS – Reactive oxygen species

h – Hours

UV – Ultraviolet

Col-1 – collagen 1

TIMP-1- Tissue inhibitor of matrix metalloproteinase-1

FDA – Food and drug administration

BDDE – 1, 4-butanediol diglycidyl ether

CaHA – Calcium hydroxyl apatite based

PLA – Poly-l-lactic acid-based

US – United States

IU – International units

CMAC – Complications in Medical Aesthetic CollaborativmL – Mililiter

NaCl – Sodium chloride

cm - Centimeter

DMEM – *Dulbecco's Modified Eagle's Medium*

PBS – Phosphate-buffered saline

G – Gauge

mm – Milimeter

AB – Alcian Blue

HAS – Hyaluronan synthases

HAPB – Hyaluronic acid binding protein

INTRODUCTION

Healthy and beautiful skin have always had a strong effect on the perception of human "well-being". While in other organs the aging process is not visible, skin gives obvious signs of aging. [1] The pursuit of reversing the effects of aging is not recent. [2] In order to develop anti-aging products is necessary to understand the mechanisms of this process. [3]

Aging is a complex process conducted by intrinsic (genetic, cellular metabolism, hormone and metabolic processes) and extrinsic (chronic light exposure, pollution, ionizing radiation, chemicals, toxins) factors. [1,4-5] All these factors lead to physiological alterations that change the skin appearance, particularly on sun-exposed skin. [1]

There is a physiological gradually loss of skin elasticity, causing sagging. Every year the total content of collagen per unit area of the skin surface declines approximately 1%. Reduction of collagen content weakens the connection between dermis and epidermis, contributing to the formation of wrinkles. On photoaged skin, glycosaminoglycans (GAGs), like hyaluronic acid, are associated with abnormal material, instead of binding with water, therefore they aren't as effective on functions like hydration of the skin. [1,3,6]

Collagen, elastin and GAGs are the three main components of the dermis associated with the aging process, thus they are subject to anti-aging strategies, from creams to fillers. [3] Fillers are products meant to be injected into the skin with the purpose of improving its physical features by soft tissue augmentation. [1] The ideal filler would be one that is long lasting, biocompatible, nonallergenic, nonmigratory, not expensive and easy to apply. Hyaluronic acid (HA) filler has numerous of these advantages in comparison with others, such as minimal immunogenicity, relative ease to use and the existence of an enzyme that can reverse eventual complications (hyaluronidase), that makes it the "gold standard" treatment nowadays for soft tissue augmentation, deep skin hydration or facial contouring. [2,4]

Considering the popularity of HA fillers and since hyaluronic acid it's also an important substance on human body, it's crucial to study the effect of exogenous hyaluronidase, used for the treatment of HA filler complications, on intrinsic hyaluronic acid on tissues, like dermis.

1. Skin

1.1. Constitution

The human skin is composed, from surface to depth, by the epidermis, dermis and the subcutaneous layer and also by its appendages, such as hair or nails. [6-9]

There are 5 layers in the epidermis that form a stratified squamous epithelium: stratum corneum, the lucidum layer, the granular layer, the spinous layer and the basal layer. [7] Keratinocytes makes up 80% of the cells in the epidermis. They migrate from basal layer, where they have full proliferation capacity, to the stratum layer, now called corneocytes, where cells become more and more flat with tight junctions, occluded by extracellular lipids, providing barrier function. [8-9] From this level where no aqueous material can penetrate, it is also the upper limit where hyaluronic acid stain on epidermis ends. [6]

On the other hand, the dermis is made up of a fibrous-collagenous-elastic tissue. [9-10] Its thickness depends on the body site, ranging between 50 and 120 μm. [11] It hosts blood vessels, nerves and sensory receptors, thus the dermis layer nourishes and supports epidermis. [9] The dermo-epidermal junction is wavy duo to the dermal papillae protrusion on the epidermis side. [7] The dermis can be divided in papillary layer, in a more superficial level, and reticular layer, the deeper one. The collagen fibers are different in each of them. In the papillary layer, type III collagen is the most abundant and their fibers are disorganized and slender, whereas on the reticular side, type I is the most common, and collagen fibers bundles are thicker and have an organized disposition, parallel to the epidermis, and intertwine with each other. [9-10]

Between cells there is an organized structure composed of glycosaminoglycans (GAGs), proteoglycans, glycoproteins, peptide growth factors and also by structural proteins like collagen and elastin which forms the extracellular matrix (ECM). [6] The GAGs' family includes 6 molecules: chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin sulfate, heparin, and hyaluronic acid. [12]

The connections that hyaluronic acid creates with other matrix proteins, such as collagen network work as a sieve, excluding some substances, and give the matrix stiffness as "shock absorver" in the dermis. [5,12-13] The skin hydration depends on the binding of water to hyaluronan molecules in dermis and epidermis. However, the maintenance of that hydration is directly related to the lipid barrier, synthesized by keratinocytes, in the epidermis, which prevents water losses. [6]

1.2. Animals skin models

Although human skin samples are the "goldstandard" for dermatology research, it is not often available. Moreover, there is an inter and intraindividual variability based on donor factors as gender, age, race and anatomical site. In this sense, there has been an attempt to find a skin animal model. The monkey and the pig are in general chosen over the rodents. Research with primates is, however, limited for ethical reasons. On the contrary, domestic pig is an animal in which skin samples are easily obtained from slaughterhouse waste. As a result, domestic pig has been commonly cited as a good model for human skin. [14]

Some studies [15-16] say that, morphologically and functionally, the pig is the animal more alike with the human skin. Some similarities as sparsely haired; not only a thick epidermis (70-140 μm) but also identical dermal-epidermal thickness ratio (10:1 to 13:1); dermis with a well differentiated papillary body and the pattern of vascularization, as observed in figure 1. Furthermore, the lipid barrier is composed by the same lipids as in man, triglycerides and free fatty acids; both species have the same time of tissue turnover and they parallel in the structure of the collagen framework and on the biochemistry of collagen itself. [11,15,17] Immunohistochemically, there were also found similar staining patterns for several antigens, namely keratins 16 and 10, collagen IV, fibronectin and vimentin. [18]

Nevertheless, there are still variations from human skin, such as: less dense vascularization in the subepidermal plexus, extensive deposition of fat below the subcutis, strong enzyme reactions in the connective tissue of the dermis and less content of elastic fibers, but still higher than in other animal species. [15] Regarding adnexal structures, pig doesn't have eccrine glands, and contrary to man, apocrine glands are localized on the skin surface. [11] This all should be considered in animal experiments, when studying the skin of the man. [15]

A study was performed on a pig to determine an animal model-based dermatological laboratory experiment for medical residents to train dermal filling techniques. The domestic pigs showed to be appropriate for the looking model. The size of the structures and the presence of wrinkles on their heads mimics the human face. In the end, the results were comparable to those obtained in humans. [19]

Even so, alternatives to animal studies must increasingly be sought to bridge human research. This is what the rule of the three Rs (reduction, refinement and replacement) used by Animal Ethics Committees is based on. Reduction stands for reducing the number of animals used for obtaining a certain quantity of information. For example, better experimental design, reduction of variation between individuals or environmental conditions are ways to achieve smaller sample sizes. Refinement principle intends to decrease the incidence and/or the severity of inhumane procedures applied to the animals which can't be replaced for this purpose. Replacement, on the other hand, was defined by the substitution of conscious living vertebrates for any scientific method employing non-sentient material. Non-sentient material can be from higher plant to micro-organisms or more degenerate endoparasites. This last rule can be subdivided in two types. Relative replacement, in which animals are still used but their suffer is believed to be inexistence. This is the case for *in vitro* studies. The second type is total replacement, meaning no animals used at all. [20-21]

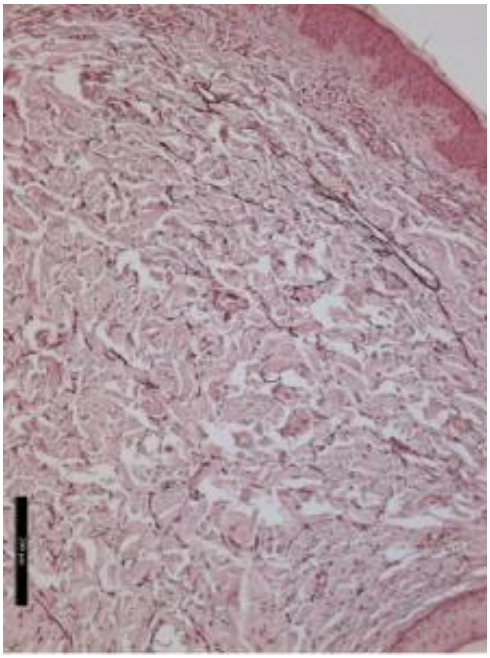

Figure 1 - Histological image of pig's skin. H&E, bar 200 μm. Reproduced from Kinney et all, 2020.

1.3. Post-mortem changes

The human body undergoes a lot of changes when it dies. Cell structures lose their integrity, endogenous enzymes start degrading the body tissues by autolysis and the presence of bacteria promotes the process of putrefaction. [22] The environmental conditions of the tissues after death, such as temperature, humidity and insect activity, as well as the individual characteristics

of each one, affects the post-mortem changes. [10] The environmental variables seem to be the main factor in cutaneous decomposition. [23]

Studies of histological changes in skin samples were performed, with different temperature conditions. With 23-37º C of temperature, the first obvious changes were observed after 9 to 12 hours after death – deformation of the basal layer of epidermis. The changes in the dermis began 18 hours after death. [7] As for temperatures of 3-25º C, the degeneration of the dermis was observed only 2 days after the time of death. [23] In another study, carried out at 4-6º C, no obvious changes were noticed in the first 24 hours, besides occasionally nuclear pyknosis in epidermis and only 72 hours after death the first post-mortem change in the dermis was observed, characterized by the swelling of the fibers. [10] As such, the decomposition of the tissues after death is faster the higher the temperatures, in which low temperatures preserves the skin tissue. [7,23]

2. Hyaluronic acid

Hyaluronic acid (HA) is a high molecular weight polymer, which is present in bacteria and all vertebrate, with a structural homology between species. This natural molecule is the ground substance in the extracellular matrix of tissues, contributing to its architecture. "Hyaluronan" is an alternative term to "hyaluronic acid", proposed in 1986, to follow the international nomenclature of polysaccharides, since it contained two sugar molecules. [24-26]

2.1. Properties:

Hyaluronic acid is a non-sulfated glycosaminoglycan made up by a repeating disaccharide structure [(1→3)-β-d-GlcNAc-(1→4)-β-d-GlcA-], composed of N-acetylglucosamine and glucuronic acid. [27] These monomers are alternately connected by β-1,4 and β-1,3 glycosidic bonds. A hyaluronan molecule can have more than 10 000 repeated disaccharides, and approximately 4 million Da of molecular mass (~ 400 dalton each disaccharide). [25] However, besides being a polysaccharide, it can also present itself as medium fragments or small oligosaccharides. [28] This biomolecule can acquire different forms depending on the pH: acid form (hyaluronic acid) and salt form (sodium hyaluronate). Under physiological conditions it occurs as hyaluronate [29], since carboxyl groups aren't associated, thus tend to bind to cations, like Na^+, and is negatively charged. [24]

In the extracellular matrix, the polymer is stabilized by interactions with hydrogen and hydrophobic bonds in an aqueous solution [30], making hyaluronic acid highly hygroscope. It

can trap around 1000 times its weight in water. [31] It is due to these hydrogen bonds, that hyaluronate acquires a helical configuration when in solution, forming a random coil. [25] This substance can entangle its chains to be cross-linked and to interact with many proteoglycans, including aggrecan and versican, forming molecular compounds. These assemblies are crucial to maintain the ECM's integrity. [26,32]

2.2. Location in the human body

HA is naturally found in several tissues and fluids, in different concentrations. It's one of the main components of the extracellular matrix of soft connective tissues, like skin, synovial fluid, umbilical cord and vitreous humor. It's present in significant amounts in embryonic tissues, being considered essential to embryogenesis. It can also be found in lung, kidney, brain and muscle tissues. [25] Although it is essentially an extracellular molecule, it can still be found inside cells, whose functions aren't described yet. To date, it's been proposed that intracellular hyaluronic acid is involved in processes like cell proliferation and inflammation. [33]

Half of the total hyaluronic acid in the body is in the skin [34], with around 400-500 μg HA/g of tissue, mainly in the dermis layer. [26] Within the dermis, HA levels are higher in the papilla than in the reticular layer. As for epidermis, extracellularly hyaluronan is found in higher quantities in the upper spinous and granular layers, whereas in basal layer is intracellular hyaluronic acid which prevails. [6]

2.3. Function

Multiple of functions have been proposed over the years, suggesting that hyaluronan is a molecule with a remarkable importance. Since it is highly hygroscope, some of its main functions are hydration of tissues and lubrification of joints. [35] In the skin, its biological functions include: water balance (in relation with lymphatic vessels), osmotic pressure and stabilization of skin structures through electrostatic interactions. [5] When extruded to the extracellular space, hyaluronic acid creates a kind of protective coat around the cells that produced it [36] and a framework along with other molecules in the ECM that allows migration of cells [35] and blood vessel formation. [37] Besides being a passive structural molecule, HA also has a huge role in cell signaling, regulating functions like cell proliferation, migration and differentiation by interacting with a number of receptors, being clusters of differentiation 44 (CD44) the most described one. This cell surface glycoprotein mediates among other physiological mechanisms: macrophage aggregation, chondrocyte pericellular matrix assembly, and leukocyte activation. [25] There are studies reporting the increase of the

expression of HA-binding receptors CD44 and hyaluronic acid-mediated motility receptor (RHAMM) as well as the increase of HA levels, in some tumors. [38-39]

The balance between synthesis and degradation ratios not only affects the concentrations of hyaluronic acid in tissues, but also the molecular weight (MW) of molecules, and consequently its mechanism of action. It has been reported opposite biological actions, whether it's a high or low molecular weight hyaluronic acid. Hyaluronic acid molecules with high MW have anti-inflammatory properties, modulating the movement of inflammatory cells. On the other hand, low molecular weight hyaluronate is pro-inflammatory, with angiogenic properties and contributing to wound healing. The difference in the MW of hyaluronic acid also has a role in pathological processes, such as cancer. This phenomenon is not fully clarified yet, but it is known that HA fragments (with low molecular mass) compete with the high MW molecules for the same binding interactions, modifying the dynamic of the biomatrix around cells, thereby decreasing their protection. [24,26] Furthermore, the response of most HA receptors depends on size, therefore molecules of different chain length will initiate different signaling cascades. [28]

2.4. Pharmacokinetics

Hyaluronic acid has a half-life of 2-6 minutes in the bloodstream and the total turnover in the adult man is 10-100 mg/day. Its distribution occurs in body tissues and intracellular fluids. In the blood, hyaluronan is eliminated mainly via liver [25], while in tissues hyaluronan can be degraded by specific or non-specific degradation systems. It is depolymerized by a specific enzyme, named hyaluronidase (HYAL), which cleaves the β -1,4- glycosidic bond between the two monomers. [40] Extracellularly, hyaluronan can also be non-specifically degraded into smaller fragments by reactive oxygen species (ROS), superoxide, nitric oxide and peroxynitrite, substances that are usually increased in inflamed tissues. [41] The resulting fragments can be internalized, with the help of CD44, to be more degraded. So, in addition to cellular signaling, HA's receptors are also involved in the degradation of hyaluronan's fragments in the ECM. [27,42]

In the skin, the half-life of epidermis' hyaluronic acid would have been expected to be greater than the dermis one, because the synthesis of keratinocytes is minor than that of fibroblasts and because, contrary to the epidermis, dermis maintain a lymphatic system which contributes to a rapid clearance of HA fragments. However, the half-time of hyaluronic acid in dermis is actually longer (less than a day) than in the epidermis (2-4h). [13] Exogenous hyaluronic acid is removed and rapidly degraded on the dermis. [6]

2.5. HA filler as an antiaging strategy

The unique physicochemical properties of hyaluronic acid, alongside with the fact that it is highly non-immunogenic, makes it a popular substance with several applications, highlighting cosmetic dermatology. [25]

With aging, there is on one hand, an intrinsic decreased in HA synthesis by structural cells and, on the other hand, a gradual degradation of hyaluronic acid by external factors such as UV radiation, which causes inevitably a decline in hyaluronan levels, thus leading to dermal dehydration and consequently formation of wrinkles in the skin. [4,6] This reduction in the skin is observed in epidermis, but not in the dermis, where the content remains constant. The reason of why it's different is unknown. Decrease on size of hyaluronan particles has also been reported, which also contributes to the skin moisture loss, characterized in aged skin. [5-6] In this sense, HA filler it's a key tool for skin rejuvenation, used as a regional volumizing filler for wrinkle and fold treatment. [2,4]

Injection of HA is believed to increase both hydration and fibroblast activation in the tissues and subsequently help the skin rejuvenation. It's also known that exogenous HA have the ability to promote the expression of collagen 1 (Col-1) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). [1]

HA fillers are approved by FDA for "mid-to-deep dermal implantation for the correction of moderate to severe facial wrinkles and fold". However, they are used too in other "off-label" anatomic locations, such as lips. [2] These fillers are produced from fermentation of *Staphylococcus equine* and then chemically modified to be more cross-linked in order to prolong their effect. The duration of the aesthetic effect of HA fillers varies between 3 to 12 months, but can go up to 1 or 2 years in long-lasting dermal fillers. [1]

The cross-linking degree is quantified by the percentage of hyaluronic acid disaccharide monomer units bound to a crosslinker molecule. It's known the higher the cross-linking, the more resistant the filler is to degradation by hyaluronidase and free radicals. The same applies with higher HA concentration, particle size and molecular weight, by increasing the biostability of the molecule. One of the main used cross-linking agents is 1, 4-butanediol diglycidyl ether (BDDE). [43-45]

There are a lot of different HA fillers depending on the indication, wanting duration of effect, recommended depth of product injection, injection technique and facial areas to be

treated. The composition differs in the particle size, cross-linking and its type, phasic structure (mono or biphasic), concentration of hyaluronic acid and presence or not of an anesthetic agent. [1]

2.5.1. Complications of HA fillers

Despite the very safe profile, there are some complications reports resulting from filler injections, which include: unsatisfactory result, delayed-onset nodules and Tyndall effect. Additionally, there may be also an intravascular injection or sidewall compression of vascular structures, causing vascular occlusion. [46]

Poor aesthetic outcome can happen due to incorrect placement, migration or excess of filler. When the filler is placed in the tissue, it starts to break down and some of its rheological properties alter, which can often change the aesthetic result, such as migration into areas of high muscle activity. [47]

The delayed nodules can be formed by a hypersensitivity reaction (type IV), a granuloma, a biofilm or a mix of the previous. They can develop weeks or months after any filler injection. [47] The Tyndall effect is characterized by a blue-gray appearance to the skin, following the filler injection into a superficial plane. The reason behind this phenomenon is the same to why veins appear blue in the skin: shorter wavelengths (blue) are more scattered than longer wavelengths (red). [46-47] Lastly, vascular occlusion may lead to granulomatous reactions, infections, tissue necrosis, visual impairment and ultimately blindness. [4,46] It's caused by an interruption of vascular supply, and it could be due to injury, compression or, more often, due to obstruction (intraarterial injection) of the artery. This condition can be recognized by the immediate severe pain, skin discoloration within a few hours, due to cutaneous ischemia, and later by skin necrosis and ulcers. [4]

All practitioners should know well the anatomy of the area they are treating. In order to prevent these occurrences, there are safety strategies that should be followed, such as: avoiding injections in an area with large blood vessels; the use of a blunt cannula instead of a needle (considering location); before injecting, pull back the syringe plunger; and inject slowly, always observing for tissue blanching. [45]

One of the main reasons hyaluronic acid fillers are currently the most used fillers in facial harmonization is because, in contrast to other fillers like calcium hydroxyl apatite based

(CaHA) or poly-l-lactic acid-based (PLA), HA has an antidote, hyaluronidase, to reverse eventual complications from filler injections, which is a huge advantage. [4]

3. Hyaluronidase

Hyaluronidase is an endoglycosidase that breaks down the hexosaminidic β (1-4) linkages. [4,48]. It is an enzyme that has a "spreading effect" on tissues, by degrading one of the major components of extracellular matrix, hyaluronic acid, as observed in figure 2. [4] At some extent, it also degrades other acid mucopolysaccharides in the connective tissue. [48-49]

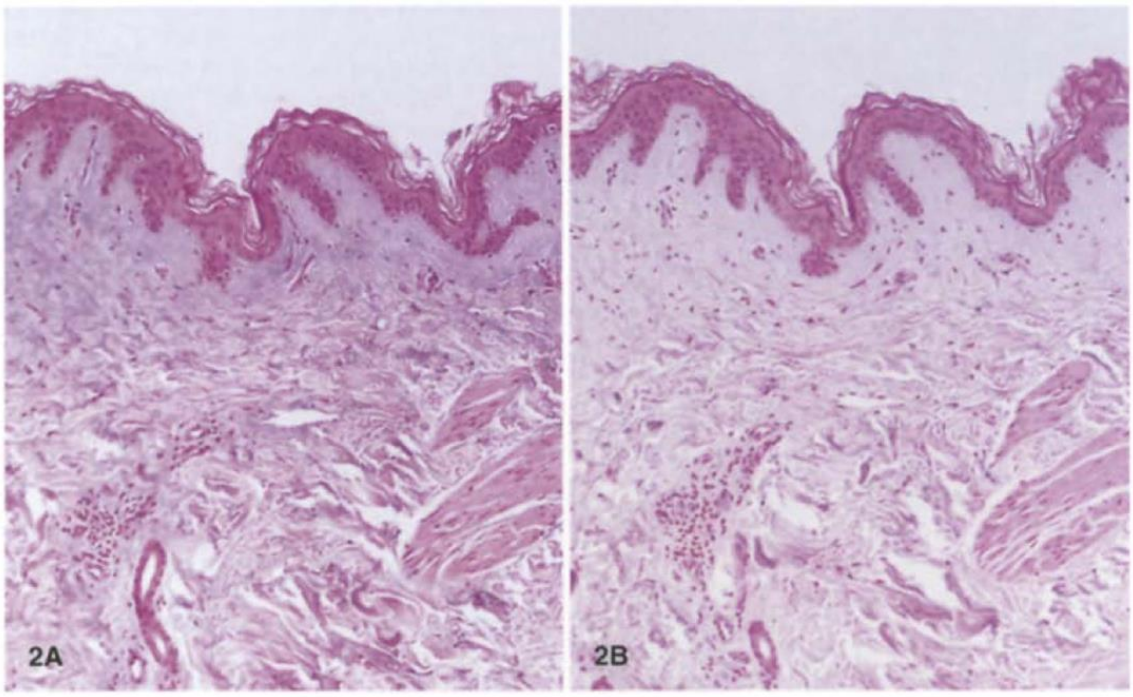

Figure 2 - Histological image of skin stained with alcian blue before (2A) and after (2B) streptomyces hyaluronidase. 50x. Reproduced from Ono et all, 1996.

It is only approved by the US Food and Drug Administration for 3 indications: subcutaneous fluid infusion; as an adjuvant to facilitate the absorption and dispersion of drugs; and to promote the absorption of contrast media in subcutaneous urography. [50] In European Union HYAL is also labeled as a promoter of reabsorption of subcutaneous hematomas. [51] Besides that, it is used off-label for several other situations. Dermal fillers attractiveness on esthetic treatments has increased the use of hyaluronidase as an essential tool for medical practitioners to correct eventual complications or unsatisfactory results. [48]

3.1. Types of hyaluronidases

Hyaluronidase can be classified according to its mechanism of action in 3 types: mammalian, leech/hookworm and microbial hyaluronidase. The mammalian hydrolyzes the β-1,4 - glycosidic linkages in HA, chondroitin and chondroitin sulfates, forming mostly tetrasaccharides. The second group are endo-β–D-glucuronidases, which produce tetra and hexasacharides, targeting the β - 1,3 glycosidic bond. These are found in leeches and some crustacean. While the previous act by hydrolysis, microbial hyaluronidase reaction is by elimination and results in unsaturated disaccharides. [49,51]

Within mammals are the human's, which are six identified: HYAL-1, HYAL-2, HYAL-3, HYAL-4, PH-20 and HYALP-1. HYAL-1 and HYAL-2 are abundant in somatic tissues, thus are the most described ones. [49] Hyaluronidase-2 hydrolyzes the HA's chain and then internalizes hyaluronic acid fragments through a membrane binding and CD44. The subsequent fragments are further degraded inside lysosomes, by HYAL-1. [42] The actions of hyaluronidase 1 extends to the plasma and it's activated in acidic pH. Hyaluronidase 3 was only found in the testis and bone marrow, where its role is unknown. PH-20 or also known as testicular hyaluronidase is found on the surface of sperm, with the purpose of degrading HA in the ovum, in order to fertilize it. [48-49]

Another classification by pH, divides hyaluronidases in acid active or neutral active hyaluronidases. The first ones are most active in pH between 3 and 4, whereas the neutral active hyaluronidases between pH 5 and 8. [51]

Hyaluronidase formulations are usually extracted from bovine or ovine or from bacteria. Bacterial forms are produced from *Streptococcus agalactiae*, but only the one from streptomyces hyalurolyticus is specific for hyaluronic acid and doesn't degrade any other GAG. [52-53] Nevertheless, bovine hyaluronidase (PH-20) is the most available one. [49,54] For medical purpose, purification of mammalian hyaluronidases is a mandatory step nowadays, to decrease the likelihood of an immune response. [48] Recently, a human recombinant hyaluronidase has been developed, which is consider safer than the rest. [53,55]

3.2. Hyaluronidase formulations in the human body

As soon as hyaluronidase is injected, it starts to degrade its substrates. So, sometimes there are immediate improvements. The effect of hyaluronidase lasts for approximately 48 hours in dermal tissues [56], although its half-life in tissues is really short. [47] When injected, its activity

in the body decreases gradually over time because of dilution, diffusion and eventually deactivation by anti-hyaluronidase activity. The anti-hyaluronidase activity is different in each tissue. [48]

The only side effects associated with injectable hyaluronidase are local pruritus and allergic reactions. Although none of them are common (less than 1%) [48], it is recommended to perform a skin test with 3 IU of hyaluronidase before injecting it. [56-57] However, this allergy test is neither valid nor reliably sensitive. [47,54,56] This doesn't apply for urgent situations, such as vascular occlusion cases, where no pre-test is performed. [58] Bees and wasps have HYAL in their poison, therefore that could work as some source of sensitization. However, the person's allergy history was shown to be irrelevant in the reaction to hyaluronidase. [48,56]

Allergic reactions are less frequent in bacterial hyaluronidase than in bovine testicular one. The reason why animal-derived hyaluronidases have more incidence of these complications is because it can be contaminated with immunoglobins and proteases, which are immunogenic. [53,59] Now the human recombinant hyaluronidase is the safest and least immunogenic form. [53]

3.3. Hyaluronidase in the treatment of HA filler's complications

3.3.1. Emergency use

For resolution of vascular complications, hyaluronidase should be administrated ideally in the first 4 hours, since within this period injection of hyaluronidase showed great effectiveness in reducing skin necrosis. After that period, the chances of success decreases and after 24 hours there's no evidence in avoiding skin necrosis. [51]

Since HYAL is able to permeate the vascular walls, there is no need for intravascular injection. Thus, the administration should be perivascular, and take into account the course of the obstructed artery. [4]

As for the dose used, Complications in Medical Aesthetic Collaborative (CMAC) have provided a guideline for the use of hyaluronidase on the management of vascular complications. CMAC recommends the injection of 1500 units of hyaluronidase with 1 mL NaCl 0,9% or 1-2% lidocaine. It's essential to cover all of the ischemic area with the enzyme, even if that exceeds 1500 units at a time. [58] A high-dose pulsed model should be adopted, meaning the area should be reevaluated after 60 minutes and in case there is still vascular compromise, the treatment should be reapplied hourly up to four times. Until resolution, daily follow up should occur. [60]

3.3.2. Elective use

In cases of overcorrection, nodules or Tyndall effect, the administration should be limited to the site of the filler injection. In order to not completely degrade the filler, it is recommended small amounts of hyaluronidase, distributed over several sessions, if necessary, to achieve the desired extent of correction. [4]

When it comes to treat an unsatisfactory result from HA injection, there are no standard concentrations. It depends on various characteristics and quantity of the filler. Fillers from different brands, with different concentrations and different physicochemical properties are differently degraded by the same amount of HYAL. [47] For example, the more cross-linked the HA, the more resistant to enzymatic degradation, so they are designed to have more longevity. [61] There is a general agreement in the literature that 5 units of HYAL is sufficient for every 0,1 mL of 20 mg/mL HA. [60] CMAC advocates "treating to effect" to be more reliable than specific doses. Still, CMAC recommends to not use concentrations less than 1500 units in 5 mL. [47] For elective complications, doses from 3 to 75 units of hyaluronidase are usually administrated. [62] Results should be assessed 48 hours after treatment and may be repeated every 48h or longer. [60]

In any case, elective or emergency, after the administration of HYAL the area should be massaged, to promote the breakdown of cross-linked HA. [47] Reapplication of HA should be performed after at least 3 hours of hyaluronidase injection. [63]

Although many studies have described the efficacy of hyaluronidase products in resolving undesirable effects of HA fillers [51,64-69], little attention has been paid to their safety. Some studies have evaluated this matter specifically regarding human recombinant hyaluronidase [70-72], although not exactly on the skin. Fewer have intended to study the same for the animal-derived hyaluronidases, which are still the most commercialized formulations. For this reason, a histologic analysis of the effects of a bovine hyaluronidase is performed in the present study. It was performed on post-mortem skin tissue of a pig, since it has been proven to be a good skin model and no greater changes occur in dermis in the first 18 hours after death, when at 37ºC. [7,14] The intrinsic hyaluronic acid was observed through the Alcian Blue staining, which has been widely used to evidence it. [63,73-75]

OBJECTIVES

The aim of this pilot study is to evaluate the time effects of two different hyaluronidase doses application on dermal intrinsic hyaluronic acid of a post-mortem pig skin.

It was hypothesized that there was no difference on intrinsic hyaluronic acid of the post-mortem pig skin with different hyaluronidase concentration over time.

MATERIALS AND METHODS

An ex-vivo experimental study was performed in the Faculdade de Medicina Dentária da Universidade de Lisboa. It was evaluated the effects of two different doses of injectable hyaluronidase on the intrinsic hyaluronic acid of pig's skin over 6 hours.

As shown in **figure 3**, there were 4 different groups: injection of 2 different hyaluronidase's doses (18,75 IU and 37,5 IU), injection of saline solution and no injections. Biopsies were taken at baseline, 1h and 6h after injections and analyzed.

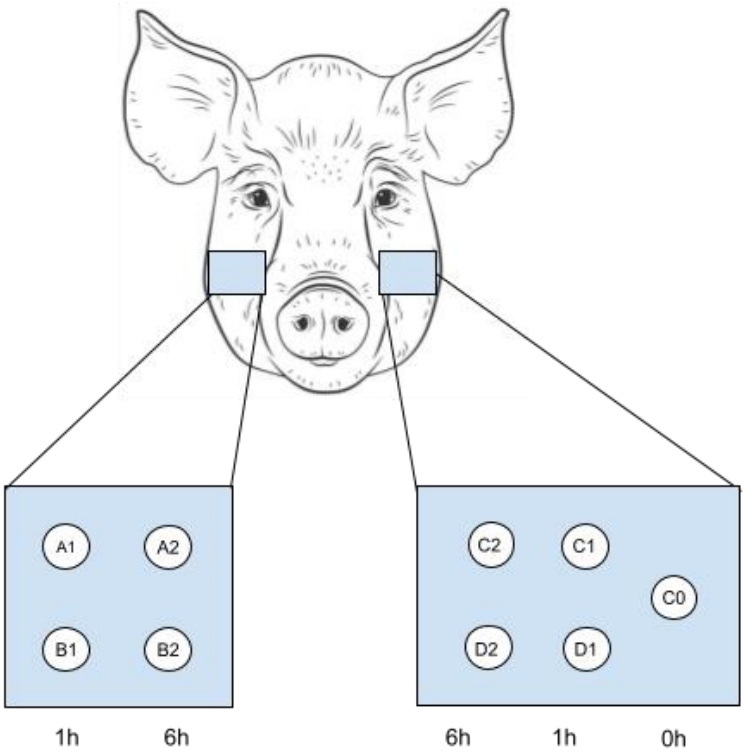

Figure 3 - Study design. 1h injection of 18,75 IU of HYAL (A1), 1h injection of 37,5 IU of HYAL (B1), 6h injection of 18,75 IU of HYAL (A2), 6h injection of 37,5 IU of HYAL (B2), Negative control group (C0, C1, C2), Positive control group (D1, D2).

1. Obtaining skin samples

The pigs from a local slaughterhouse (Sicasal, Mafra, Portugal) were subjected to a 98% carbon dioxide chamber in order to first lose consciousness. Then they were killed through a cut in the carotid artery. Two symmetrical skin samples were collected right after pigs' death

with an area of 5.5 x 10,5 cm each, using a scalper with a 15 blade, dissecting forceps, detacher and metallic ruler. The skin was priorly disinfected with chlorhexidine (DENTAID®, Barcelona, Spain), followed by 70% isopropanol (Wells®, Albufeira, Portugal) 3 times. Most of the hair was then shaved. The pieces were immediately placed in containers with Dulbecco's Modified Eagle Medium (DMEM) (Biowhittaker®, Walkersville, Maryland, United States), whose volume was enough for each piece to be immersed, at 37º C, in order to maintain the tissue viability. (76)

2. Points marking

For injections to be performed, the skin pieces were removed from the containers and washed in phosphate-buffered saline (PBS) (VWR®, Radnor, Pensilvania, EUA) 3 times, for 5 minutes each. For the injection points to be 2,5 cm apart from each other to avoid interference of reagents and 1,5 cm from the margins, 12 suture points were made 1 cm away from these and 0,5 cm from the margins, to serve as a reference for biopsy.

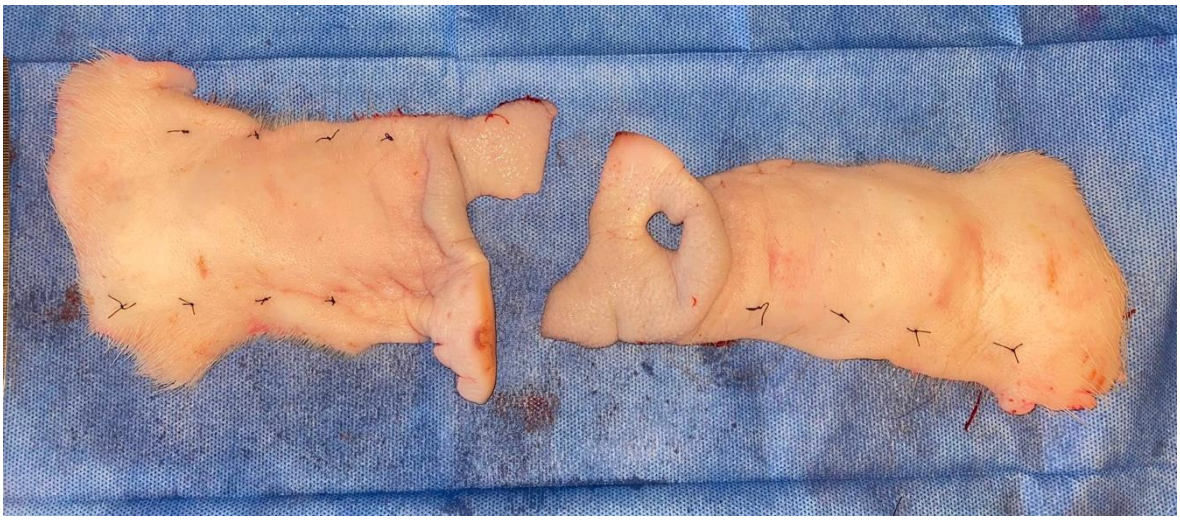

Figure 4 - Skin samples marked

3. Injection performance

The BCN hyaluronidase (Institute BCN®, Barcelona, Spain) available on the market was chosen. Two different hyaluronidase concentrations (375 IU/mL and 187,5 IU/mL) were achieved by the dilution of a 1500 IU vial in 4 mL and 8 mL of saline solution, respectively. Saline solution was also used as positive control of HYAL.

Prior to any injection, the skin was disinfected with a chlorhexidine solution. All injections were performed by a dentist doctor experienced in facial harmonization. Every injection was followed by a massage of the area. Each row of marks corresponds to a different group so 0,1 mL of each hyaluronidase's concentration was injected in a row of points, 0,1 mL of saline solution was injected in another row of points (positive control) and on the last row of points nothing was injected (negative control). All solutions injected were slowly administrated with a 28G needle with 12 mm and 1 mL syringe, perpendicularly to the skin into the dermis. [51]

The pieces were then placed again into the same containers as previously, in the same conditions, until biopsied.

4. Skin biopsies

Right after injections, a biopsy of the initial moment from negative control group (no injections) was taken. Then, all groups were biopsied 1 and 6 hours after injection, in a way that each point of each group had a different time of HYAL application. Between each biopsy time the pieces were always placed in their conservation method.

Biopsies were performed by "punch" with a diameter of 4,5 mm, in order to include injection sites and surrounding tissues of the marks. Each sample was placed in a 2 mL cryopreservation tubes with 4% formaldehyde (Panreac AppliChem®, Chicago, USA) and sent for histopathological analysis.

5. Histological processing of the samples

The skin samples were processed histologically by a student with the help of an expert in pathological, cytological and thanatological anatomy, from the Faculty of Dental Medicine of University of Lisbon.

All samples were fixated for 4 hours, followed by dehydration, with increasing amounts of alcohol (70% for 1 hour, 96% for 45 minutes and then 1 hour, and absolute ethanol for 45 minutes and then 1 hour). Different alcohol solutions were reached through mixing of absolute ethanol (Honeywell Riedel-de Haën®, Seelze, Germany) and water. Diaphanization was then performed using first a mix of xylene (VWR®, Radnor, Pensilvania, EUA) with 100% alcohol in 1:1 ratio, for 1 hour, and then xylene only, 1 hour, 2 times. Since the biopsies were thick, in order to guarantee the dehydration and diaphanization of the pieces, the previous steps were repeated twice. Three changes of molten paraffin (VWR®, Radnor, Pensilvania, EUA), 45

minutes to 1 hour each, was used for embedding and finally cross skin sections were made with 3 µm, using a microtome (Leica®, Wetzlar, Germany). [77-78]

Deparaffinization was achieved with xylene, for 10 minutes at room temperature. Afterwards, the slide is passed through decreasing solutions in alcohol (absolute ethanol, 96% ethanol, 70% ethanol) for 2 minutes each and finally through rinsed water for 1 minute as well. The hydration allows penetration of reagents into cells and tissue elements. [78]

6. Coloration technique

For the analysis of each biopsy, two slides were used: one stained with Hematoxylin & Eosin for morphometry and the other with Alcian blue to evidence hyaluronic acid. [75,78]

For Harris's hematoxylin solution 6g of Hematoxylin was added in 60 mL of absolute alcohol and 120g of potash alum to 1200 mL distilled water. After boiling the second solution, the first solution was added. Three grams of mercuric oxide were added gradually. The mixture was kept in cold water until a dark purple color appeared and then it was added 48 mL of acetic acid. [78-79]

The slide was then stained, with Harris hematoxylin for 10-15 minutes. After rinsing in tap water for 10 seconds, 2 times, and then for 5 seconds, in order to differentiate the stain a weak acid alcohol (70% alcohol with 0.5–1% hydrochloric acid) is used for 5 seconds to remove non-specific background staining. Right after, the slide is washed again in tap water for 10 seconds, 2 times, and then for 5 seconds. [78-79]

A 0,5% eosin Y solution was then applied for 10 seconds. The section is now passed through increasing amounts of alcohol (96% alcohol 2 times and 100% alcohol) for 2 minutes each and then for two xylene baths 2 minutes each, for dehydration and clearing, respectively. Finally, a coverslip glass was placed after the application of a thin layer of entellan (Merck KGaA®, Darmstadt, Germany) mountant. [78-79]

For the alcian blue solution (pH 2,5) 1g of Alcian blue (8GX) was dissolved in 100 mL of a 3% acetic acid solution. [78,80]

The slide was placed in 3% acetic acid solution for 3 minutes and then stained in alcian blue solution (pH 2,5) for 30 minutes. Right after, it was washed in running tap water for 14-20 minutes. A counterstain was achieved with nuclear fast red solution (5g of alumium sulfate, 100 mL deionized water and 0,1 g nuclear fast red), followed by a 1 minute rinse. Lastly,

dehydration and clearing were performed in the same way as H&E stain. The same medium was used for the mounting. [78,80]

7. Histological evaluation

The visualization of the stained slides was possible with an optic microscope (Leica®, Wetzlar, Germany). The analysis of the slides was performed by a student.

The slides were photographed using DinoCapture 2.4 software for Mac (Dino-lite Digital Microscope, Taipei, Taiwan).

RESULTS

Histological analyses were conducted on samples of porcine skin, which were subjected to different experimental conditions. The samples were observed and analyzed by a student with help of an experienced professional in the field. This collaborative approach ensured reliable and accurate results.

To assess possible postmortem changes, a group without injections was performed at baseline, illustrated in **figure 5**. The results illustrated in **figure 6** and **7** aimed to evaluate the effects of the injection of 18,75 IU and 37,5 IU of hyaluronidase, respectively, on intrinsic hyaluronic acid in dermal tissue, after 1 hour. For comparative purpose, two control groups were employed: a group without injections as the negative control (**Figure 8**) and another group subjected to saline solution injection as the positive control (**Figure 9**).

C0

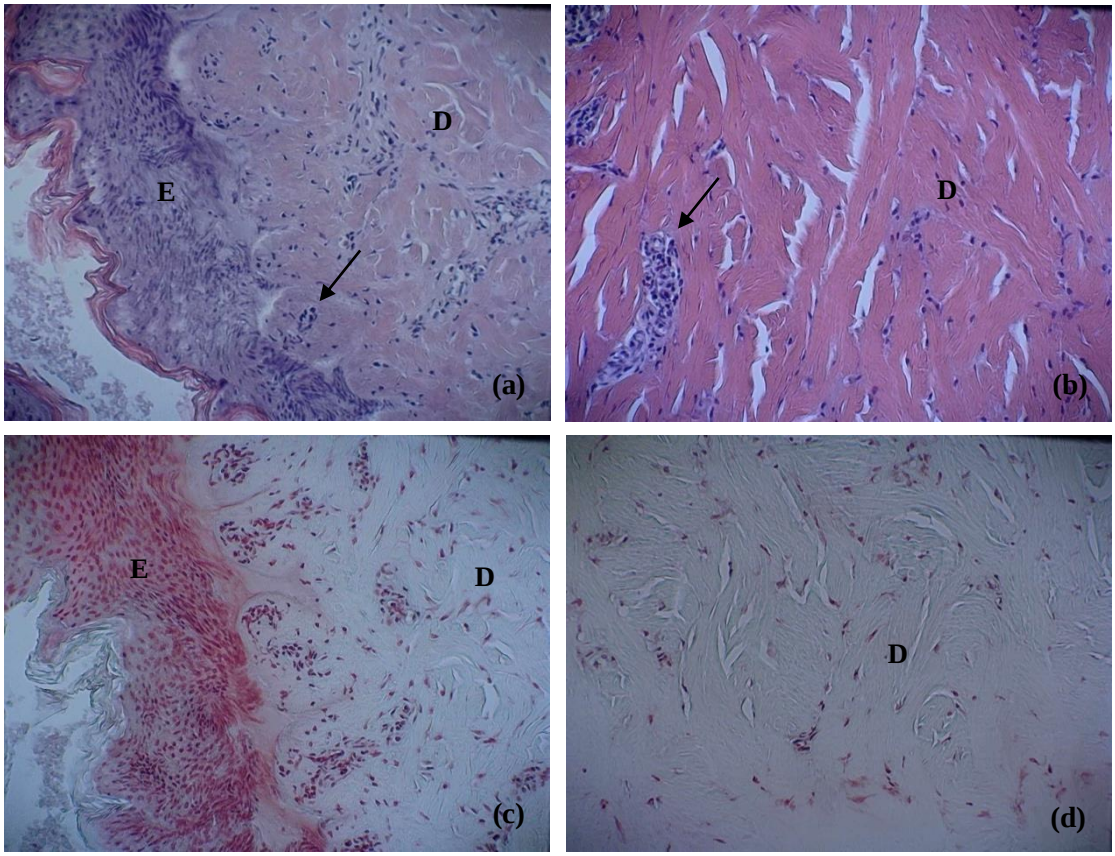

Figure 5 – Histological findings of porcine skin of negative control (no injections) at baseline, 10x objective magnification (a, b - H&E; c, d – AB). E – Epidermis. D – Dermis. Arrows indicate inflammatory infiltrate.

A1

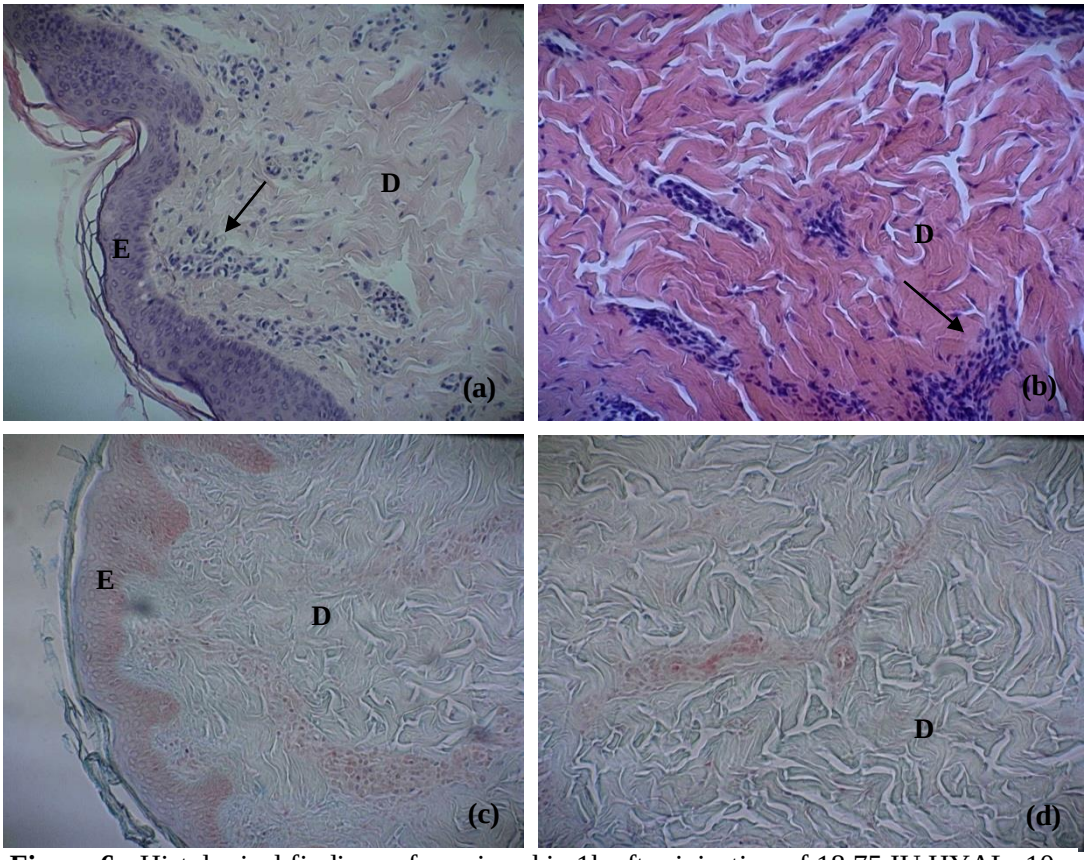

Figure 6 - Histological findings of porcine skin 1h after injection of 18,75 IU HYAL, 10x objective magnification (a, b - H&E; c, d - AB). E – Epidermis. D – Dermis. Arrows indicate inflammatory infiltrate.

B1

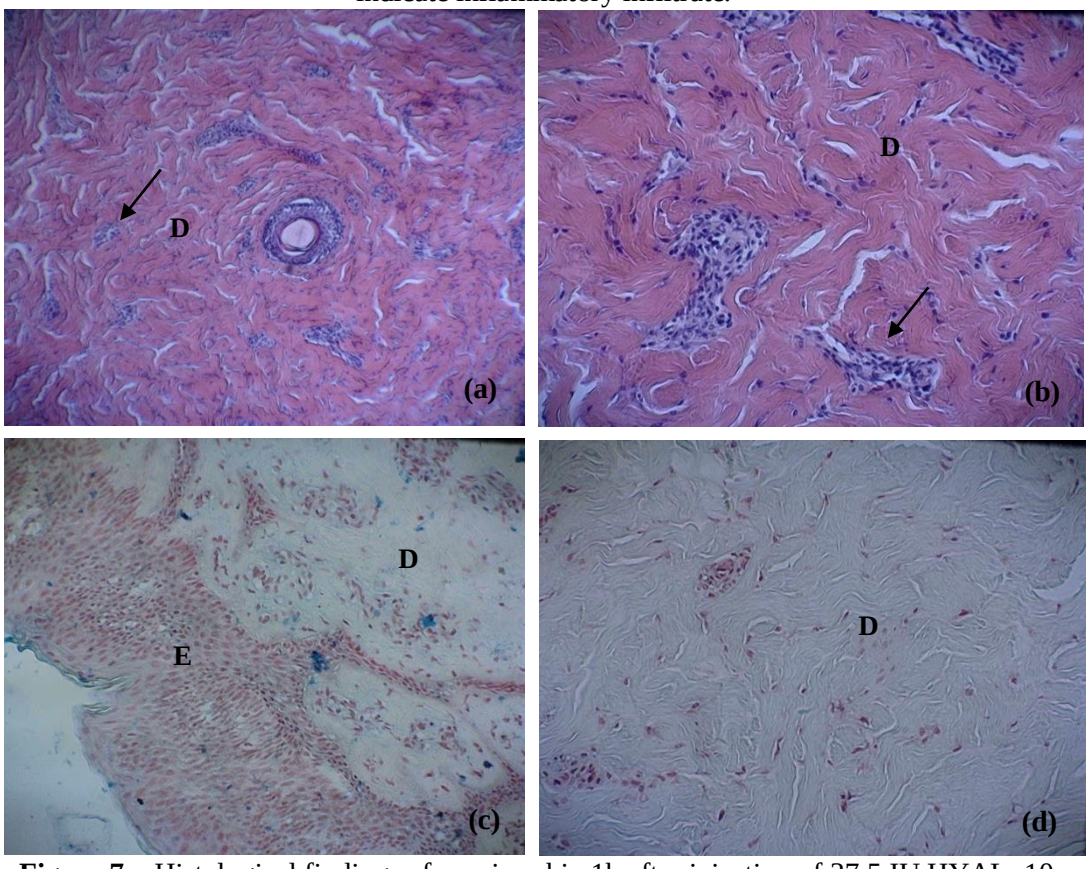

Figure 7 – Histological findings of porcine skin 1h after injection of 37,5 IU HYAL, 10x objective magnification, except (a) with 4x (a, b - H&E; c, d - AB). E – Epidermis. D – Dermis. Arrows indicate inflammatory infiltrate.

C1

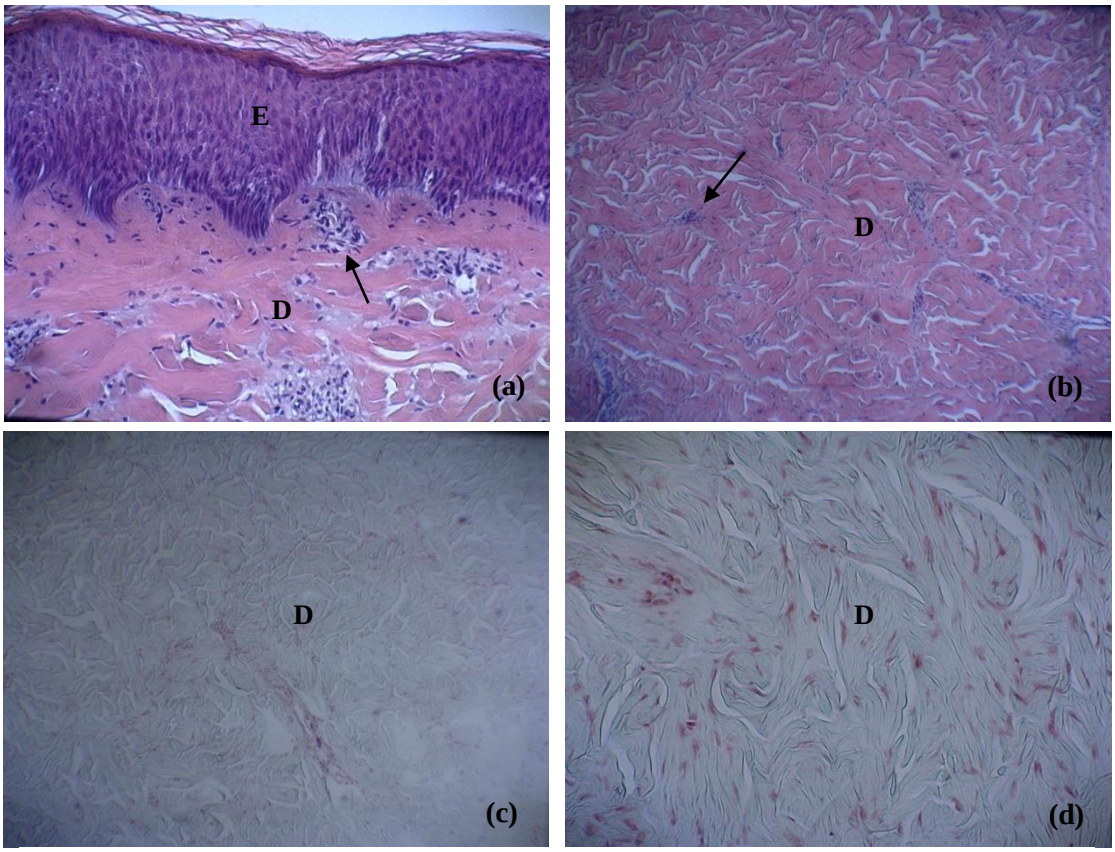

Figure 8 – Histological findings of porcine skin of negative control (no injections) after 1h, objective magnification of 10x ((a) and (d)) and 4x ((b) and (c)) (a, b - H&E; c, d - AB). E – Epidermis. D – Dermis. Arrows indicate inflammatory infiltrate.

D1

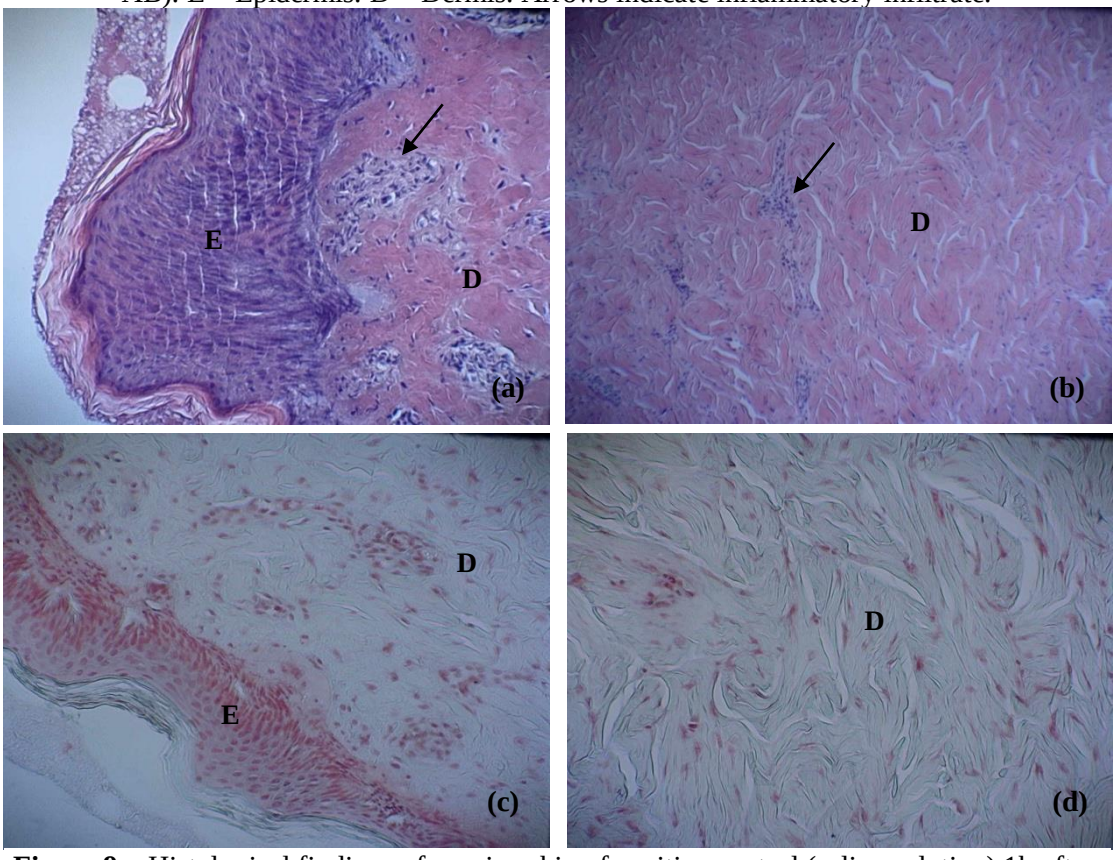

Figure 9 – Histological findings of porcine skin of positive control (saline solution) 1h after, 10x objective magnification, except (b) with 4x (a, b - H&E; c, d - AB). E – Epidermis. D – Dermis. Arrows indicate inflammatory infiltrate.

After injection performance and before biopsy, no macroscopic differences were seen between groups and over time.

The control biopsy at baseline was primarily conducted to assess the condition of the tissue at the start of the experiment. Upon histological examination, the tissue architecture was found to be well-preserved in the initial control group (C0). This is demonstrated in **figure 5**, where the epithelium appears intact without any pathological alterations. (**a** and **c**), along with the normal organization of the dermis (**b** and **d**), that included intact adnexal structures. Additionally, the subcutaneous tissue, such as muscle and adipose tissue, exhibited a conserved structure, although specific details are not provided in the available data.

At 1 hour, the skin samples of the different groups also demonstrated preservation of tissue architecture, as seen in **figure 6, 7, 8** and **9**. The integrity of the epidermis, with appropriate thickness and distribution of its layers and maintenance of the corneal layer, is observed in both groups of hyaluronidase injection (**figure 6 – a, c; figure 7 – c**), as well as in control groups (**figure 8 – a; figure 9 – a, c**). Although the epidermis was not visible in some of the slides examined (1 hour after injection of 37,5 IU HYAL stained with H&E and 1 hour after of negative control stained with AB), there is evidence of tissue preservation by its presence in the other staining of each of these groups. The dermis appears with a normal organization, applying to both the superficial and deep dermis, observed immediately beneath the epithelium and in **figure 6, 7, 8 and 9 – b, d**, respectively.

In the observed slides, both the hyaluronidase and saline solution injection groups exhibited disruption of the epithelium and dermis corresponding to the injection site, compared to the negative control group.

A lymphoplasmacytic inflammatory infiltrate was observed in all histological samples examined, with similar intensity and distribution.

Furthermore, the intensity of Alcian blue staining, used to visualize hyaluronic acid, was similar in all analyzed samples both at the initial time point (**Figure 5 – c, d**) and after 1 hour of injecting different concentrations of hyaluronidase (**Figure 6 and 7 - c, d**), saline solution (positive control) (**Figure 9 – c, d**), and no treatment (negative control) (**Figure 8 – c, d**). The connective tissue was homogeneously stained, showing a light blue color. Cell nuclei appear stained red.

DISCUSSION

The present study investigated the dose-effect of a bovine hyaluronidase on dermal hyaluronic acid, after dermal injection, equivalent to the clinical use of this product. Similarities in the skin between humans and pigs regarding structure and vascularization network allowed reliable prediction of morphological and cellular changes as well as histological behavior of the injected hyaluronidase in humans. [14-17,19]

According to the manufacturer, the BCN hyaluronidase is indicated for the treatment of cellulite in the skin, claiming it to eliminate fluid retention and improving cellular exchanges of triglycerides accumulated inside adipocytes. Hyaluronidase is known to act both on epithelium and connective tissue, depolymerizing glycosaminoglycans, like hyaluronic acid and chondroitin sulfuric acid, responsible for water retention and playing a role in adipogenesis, therefore, reduces the fibrous edema characterized of cellulite. [81, 82] Besides the original indication, like any other hyaluronidase, this formulation is also used off-label by a lot of practitioners for the treatment of HA filler's complications.

In the literature there is a wide range of doses of HYAL applied for degrading HA fillers, from 1,5 to 2000 IU [47-48,51,54,56,62,67,83], and some studies don't even specify. This particular hyaluronidase is not mentioned in the literature so there was no reference for the amount of HYAL that should be applied for the indication of interest. In spite of this, we opted to comply with the manufacturer's instructions, leading to a concentration of 375 IU of HYAL/mL and decided to make a further concentration, 2 times less concentrated (187,5 IU/mL), for comparison of the effects. The amount of volume injected was based on previous studies. [54,62,84]

Only 5 biopsies out of 9 were analyzed, therefore it was not possible to study the effects over 6 hours, with only histological analysis after 1 hour of HYAL application and of the biopsy at the initial moment. The remaining biopsies are still being processed and waiting to be analyzed.

The pieces were stored in DMEM, which in a previous study proved to keep the cell viability of fresh pig's samples for 18h at 25ºC. [76] Although this experiment was performed at 37º C, in order to mimic the human body conditions, this conservation medium was able to conserve the tissue structure at 1 hour, since there was proper organization of various histological components, such as the epidermis, dermis, and subcutaneous layers in all groups.

This indicates that the biopsy procedure and experimental conditions, including the presence of hyaluronidase, maintained the overall architecture of the skin tissue. This is in agreement with Salinas-Alvarez et all [83], which observed that even with higher doses of the ones we used in this study (75 IU, 200 IU and 500 IU) there was still preservation of the skin morphology, with correct organization of the fibers of collagen and absence of inflammatory infiltrate, suggesting that high doses do not affect the dermis or epidermis. This finding validates the present methodology for the skin model chosen.

The histological examination revealed similar inflammatory infiltrates in all groups, suggesting that both hyaluronidase and saline injections did not induce an inflammatory response. However, compared to the negative control group, the groups that received injections exhibited dysruption of the epithelium and connective tissue at the injection site, indicating some degree of trauma, although it did not elicit a tissue-level response. Thus, the presence of an inflammatory infiltrate could be due to the tissue manipulation during the experiment or biopsy procedure that uniformly affected all samples or could just be a basal tissue response of the pig. Nevertheless, doses from 50 IU have produced an increase number of eosinophils with increase of the dose (up to 2000 IU), as well as clinical signs of inflammation, in a similar study. [85]

In the same study one of the objectives was to evaluate the correlation between hyaluronidase dose and degradation of intrinsic HA in human skin. They reported that doses above 300 IU of HYAL cause a decrease in tissue HA. However, this was clinically evaluated by tissue resistance and clinical appearance, since the staining used (H&E and iron staining) was not sensitive enough to detect changes in hyaluronic acid. [85]

Alcian blue at pH 2.5 stains HA and other GAGs, through cationic dyes. Thus, this histochemical procedure is nonspecific and often underestimates the hyaluronic acid content. Even so, the stain intensity reflects in general the hyaluronic acid content and its distribution, thus it is commonly used for this purpose. [75,86] In addition, immunohistochemistry can also be employed to visualize hyaluronic acid, like some studies report [87,88], however, this technique is relatively expensive and not commonly used due to its cost.

In the present study, no differences were found between groups when stained with Alcian blue. This suggests that there were no apparent differences in the quantity or distribution of hyaluronic acid between the initial moment and 1 hour, when in the presence of hyaluronidase

up to 37,5 IU of dose. These results deviate from the anticipated outcomes of a recent research that studied the effects of another bovine hyaluronidase on structural cells and HA-metabolism in the skin. They found that low concentrations of HYAL (0,015 IU/mL) cause a significative increase on hyaluronan synthases (HAS) activity in comparison with higher ones and consequently led to an accumulation of hyaluronic acid, whereas 15 IU of HYAL/mL led to a reduction in HA levels. [77] In this way it would be expected that the chosen concentrations (375 IU/mL) would have caused a decrease in the hyaluronic acid content. However, the study does not mention the amount injected, so we do not know the absolute doses used and therefore comparisons can't be made. Even so, the discrepancy between the findings of the mentioned study and my research may also be attributed to the different methods employed to assess hyaluronan levels. While the study utilized immunofluorescence with hyaluronic acid binding protein (HABP), which has specific affinity towards HA, my evaluation relied on the Alcian blue staining method, which was analyzed qualitatively. Therefore, the present method may not be sensible enough to detect subtle variations in HA levels like the mentioned quantitative method. Additionally, there could be the presence of GAGs sulfate that it's known to have an inhibitory activity on bovine hyaluronidase. [89]

The depolymerization of endogenous HA involves mostly enzymatic degradation by hyaluronidases but also degradation by free radicals. [90] When it comes to enzymatic degradation of HA in ECM, it is influenced by a lot of factors, such as depolymerization potency, steric hindrance, the catalytic mechanism and the substrate specificity of the enzyme. [89] There is a wide variety of hyaluronidase products with different origins, properties, concentrations and inevitably different enzymatic activities. [63,85] Therefore, a different hyaluronidase may yield different results as it may have different effects on the skin. Hence, this assessment must be done on a case-by-case basis.

Besides, the evaluation of the amount of endogenous hyaluronic acid is not the only parameter that determines the safety of a product, neither the short-term tissue response, of which it's known that HA levels return to normal after 15-20 hours of hyaluronidase injection. [47]

The results from the present study must be analyzed carefully, considering some limitations of the experiment. Although the pig is considered a good surrogate for studying human skin, there are still intrinsic differences between them, that doesn't allow completely extrapolation of data. Furthermore, this study was performed in an *ex vivo* model. After death, there is

termination of capillary circulation, so among other factors, systemic circulation only exists in *in vivo* systems. Thus, in this case there was no perfusion of the tissues, which influence a lot of their properties, such as low stiffness due to the absence of the pressure given by the blood flow, but especially, without systemic circulation, the clearance of hyaluronidase, that would normally occur, decreases, thereby prolonging its life in the dermis. [91]

Despite having a small sample, this study highlights the gap in our knowledge about injectable hyaluronidase and the effects it may have on tissue homeostasis regarding intrinsic hyaluronic acid, especially at high concentrations. Future studies should consider evaluating this relationship, but in the presence of an HA filler, since this is what actually happens in clinical reality - the need for hyaluronidase injection means the prior placement of a hyaluronic acid filler. In this way, it would be evaluated if the degradation of intrinsic hyaluronic acid changes in the presence of a HA filler, that is, if exogenous hyaluronidase has a preference for one of the HA substrates (endogenous or exogenous), knowing that fillers are specifically designed to resist enzymatic degradation. Upcoming studies should extend the time of hyaluronidase application to see its effect on a long-term analysis.

Nevertheless, these findings confirm the suitability and validity of the chosen skin model and the methodology employed in this study, so it serves as a starting point for further investigations.

CONCLUSION

The increasing popularity of hyaluronic acid fillers has led to a growing need for the use of injectable hyaluronidase. Despite the widespread utilization of hyaluronidase as an adjunct treatment for HA filler-related complications, there remains a significant gap in our understanding of its safety profile. As a result, studying the safety of this enzyme has become essential in the field. The intrinsic hyaluronic acid in the dermis plays a vital role in various physiological processes, making it of great importance to investigate the effects of hyaluronidase on its levels.

Through this study, we aimed to contribute to the understanding of the impact of injectable hyaluronidase on intrinsic hyaluronic acid of the dermis. The absence of observed histological changes through the uniformity of Alcian blue staining indicates the promising potential of injectable doses hyaluronidase between 18,75 IU and 37,5 IU as a safe intervention, for the time analyzed (1 hour).

As the study has some limitations and a relatively small sample size, it underlines the need for further research and larger-scale studies to fully evaluate the effects and long-term impact of hyaluronidase on the tissues, providing practitioners with evidence-based guidelines for its optimal and secure implementation.

Nevertheless, this pilot study shows the suitability of the porcine model and the effectiveness of the methodology employed on it so it serves as a valuable starting point for future histological investigations.

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