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Histopathological Assessment of the Safety and Efficiency of Hyaluronic Acid Filler Augmentation in Rats' Labial Dermal Matrix (Histological, Histochemical & Immunohistochemical Study)

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Aim: to assess the safety and efficacy of HA in restoring the labial volume and shape while maintaining its structural integrity.
Materials and methods: 30 rats were equally divided into two main groups; Group I (Control): Included fifteen adult rats injected subcutaneously with saline. Group II: experimental groups, included fifteen rats whose lower lip mucosae were injected with 0.02 ml HA filler. Each of the control and experimental groups were equally subdivided into three subgroups, Subgroup A: sacrificed 7 days after injection. Subgroup B: sacrificed 14 days after injection. Subgroup C: sacrificed 28 days after injection. Lip specimens were processed for routine H&E examination, histochemical examination using Alcian blue & Picro Sirious Red stain, as well as Immunohistochemical assessment using anti-cox2 .
Results: experimental groups displayed more vacuolated epithelial cells, more inflammatory cell infiltration, more cox-2 positive cells and more collagen hyalinization within the connective tissue .
Conclusion: HA filler induced a moderate foreign body reaction whose intensity increased with time.

Keywords: Hyaluronic acid filler, labial demal matrix, foreign body reaction.

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Introduction

Age-related facial volume reduction, physical, hereditary conditions, or even accidents may lead to serious alterations in appearance .**Error! Reference source not found.** The lip is a critical area when it comes to facial aesthetic enhancement. Both lips have a major role in facial appearance. Error! Reference source not found. Ideally, having full lip with a well-defined vermilion border is the ultimate goal in facial aesthetics. Moreover, the appropriate balance between upper and lower lips is crucial from an aesthetic point of view.Error! Reference source not found. The use of a bio-compatible filling material for superficial and deep soft tissue enhancement is usually used for rejuvenating the aging face.Error! Reference source not found. Although dermal fillers are broadly considered as safe and well-tolerated esthetic tools, the occurrence of unfavorable bodily reactions remains a prognostic concern. Error! Reference source not found.

Ideally, fillers should be safe (non-allergenic, non-carcinogenic), stable, easy to use and store, inexpensive, biocompatible, show little or no adverse reactions, have no migration within the targeted site, be long-lasting with slow degradation within the body, have minimal recovery period, and minimal inflammatory response. Error! Reference source not found.-Error! Reference source not found.

Dermal fillers are classified into natural fillers and synthetic fillers. Natural fillers are such as hyaluronic acid (HA), human-derived collagen, bovine collagen, calcium hydroxyapatite, and poly-L-lactic acid. while synthetic fillers are such as silicone, polyacrylamide, polyalkylimide, and polymethyl methacrylate (PMMA).**Error! Reference source not found.**

To date, HA is still the most favorably used material for lip augmentation.**Error! Reference source not found.** HA is a polysaccharide naturally present in the dermis. Its great capacity to bind water helps in hydration and maintains skin tone .Error! Reference source not found.

Unfortunately, the vast increase in filler usage was accompanied by an increased liability to adverse reactions occurrence, which could be attributed to the injection technique or chemical composition of the fillers.Error! Reference source not found. Early complications might also advance into a hypersensitivity reaction, sepsis, skin discoloration, vascular occlusion, and contour irregularities .Error! Reference source not found.; Error! Reference source not found.

Few histopathological examinations were performed on animals and human volunteers to assess HA safety. Some of which displayed the injected material surrounded by a slight to severe lymphohistocytic infiltration accompanied with plasma cells, and a variable amount of macrophages and mast cells .Error! Reference source not found.

Cyclo-oxygenase 2 (Cox-2) is the active form of the cyclo-oxygenase enzyme, which is expressed by inflammatory cells, particularly macrophages. Expression of Cox-2 within a tissue denotes a foreign-body response in this tissue .Error! Reference source not found.

Hence, it was greatly appealing to histopathologically and immune-histochemically verify the long-term safety and efficiency of HA filler on labial tissues.

Materials and Methods

Experimental Animal Model

All experimental procedures followed the protocols of the Ethics Committee of the Faculty of Dentistry, 6th of October University, Egypt. (Approval number: RECO6U/17-2023). Thirty adult male albino Sprague-Dawley rats (obtained from Theodor Bilharz Research Institute, Imbaba, Egypt), weighing between 200–250 g, were used in this study. They were housed in ventilated metal mesh cages (5 per cage) in a controlled environment with a 12-hour light/dark cycle at a regulated temperature

between 22-23°C and humidity between 55-65%. They were fed a standard diet of rat chow and had free access to drinking water.

Experimental Design

Rats were randomly divided into two main groups: **Group I** (Control) included fifteen adult rats injected subcutaneously with equivalent volumes of normal saline. These rats were fed and kept under the same conditions as the test rats and were sacrificed in parallel with those rats. *Group II* (experimental group) consisted of fifteen rats whose lower lips were injected with 0.02 ml of HA filler. Each of the control and experimental groups were equally subdivided into three subgroups according to the date of sacrifice. *Subgroup A:* in which five rats were sacrificed 7 days after injection. *Subgroup B:* in which five rats were sacrificed 14 days after injection. *Subgroup **C:*** in which five rats were sacrificed 28 days after injection.

Drug (Hyaluronic Acid Filler: HA)

DERMAFILL is a monophasic biodegradable fourth-generation injectable filler of a non-animal origin. It was purchased from (SBSMED, France).

Injection of the Filler Material:

Anesthesia was performed using an intraperitoneal injection of diazepam (0.05 ml/100 g) and ketamine hydrochloride (0.1 ml/100 g). After sedation, rats' lower lips were pulled out using tweezers to expose the labial mucosa. Using a disposable insulin syringe, 0.02 mL of the filling material was injected with the needle inclined as parallel as possible to the mucosa, 7 mm deep (to reach the dermal matrix), standardized by an endodontic silicone stop. The same dose of filler was administered to all animals, and they received injections at the same anatomical sites. The filler was injected slowly into the dermis, via the linear threading technique. Error! Reference source not found.

Histopathological Examination

According to experimental procedures, rats were sacrificed by an overdose dose of anesthesia via intravenous injection of thiopental sodium (57.8 mg/kg) .Error! Reference source not found. Specimens from the lower lip were obtained and kept in 10% neutral buffered formalin. Specimens were dehydrated in a series of alcohol, cleared in xylene, and embedded into paraffin blocks at the Pathology Department, Faculty of Medicine, Ain Shams University. They were then processed for routine histological examination with H&E, and Picro Sirius Red (PSR) for histochemical visualization of collagen types I and III in sections. Intact collagen type I fibers stain intense red, and type III stains greenish in color Error! Reference source not found., and Alcian blue (pH 2.5) staining was used for histopathological visualization of the residual amount of HA filler in tissues as it appears basophilic and is also described as alcianophilic.Error! Reference source not found., Error! Reference source not found. Evaluations included the collagen density and fibrosis around the injection site, as well as the qualitative and quantitative histologic evaluation of the local tissue effects and inflammatory response. Error! Reference source not found.

Immunostaining

Paraffin-embedded sections were mounted on slides, dewaxed in xylene, rehydrated in a graded ethanol series, and prepared for immunoperoxidase staining according to standard procedures. Primary antibody of Anti-Cox2 (Rabbit Anti-COX2 / Cyclooxygenase 2 antibody, ab6665). After staining with the primary reagent for 1 hour at room temperature, sections were washed, incubated with a biotinylated affinity-purified secondary antibody (Goat Anti-

Rabbit IgG H&L (HRP) (ab205718)) for 30 min at room temperature, washed, and treated with avidin-biotin-peroxidase for 30 min at room temperature. Then sections were washed in buffered saline and then incubated in a solution containing 0.05% 3,3 diaminobenzidine (Sigma Aldrich, Switzerland) and 0.03% H_2O_2 in phosphate-buffered saline at room temperature. All sections were examined under a microscope (Olympus microscope, Japan) using the appropriate filters.

Digital Morphometric Study

Slides were photographed and analyzed at the (Central Research Unit of MSA university, Egypt) using the color image analysis system which included: Digital microscope Leica DM3000 LED S.N 346986, camera DFC295 S.N 0705530414 Made in Germany, and Leica Qwin image processing and analysis software. Part no 872705 Version V3.5.1, Leica microsystems LTD .CH 9435 Heerbrugg (Switzerland).
Two slides from each rat were prepared, and five random fields from each slide were analyzed. Measurements included the number of inflammatory cells within CT, as well as the number of immuno-positive cells in the C.T.

Statistical Analysis

Statistical analysis of the histomorphometric data from the current study was performed using SPSS software. The Shapiro-Wilk test of normality was used to test the hypothesis of normality for all continuous variables. The analysis of variance (ANOVA) test was used to evaluate the statistical significance of each parameter within the filler groups, followed by the Tukey-Kramer post hoc test to analyze the statistically significant results. P-values ≤ 0.05 were considered statistically significant. Unpaired T-test was used to evaluate the statistical significance of each parameter between the control group (after 28 days) and the filler group (after 28 days).

Results

H&E Results

Control Groups

Subgroup IA (Control at 7 Days)

Histopathological examination of this group (gp) showed normal architecture of both the epithelium and lamina propria on the mucous membrane side as well as on the skin side of the rat's lip. The mucous membrane side presented intact thick keratinized stratified squamous epithelium with some clear cells (nonkeratinocytes) at basal and parabasal levels. Some vacuolated epithelial cells were detected at various layers. The sub-epithelial connective tissue (CT) showed abundant well-formed collagen fiber bundles, which were interspersed with numerous fibroblasts. Blood vessels of variable sizes were also evident within CT. Rare presence of inflammatory cells was noted, mainly close to blood vessels. (Figure 1a). The skin-side showed well-developed hair follicles, as well as sebaceous glands (Figure 1b).

Subgroubs IB (Control at 14 days) & IC (Control at 28 Days)

These groups displayed almost the same histological features as the previous gp.

Experimental Groups

Subgroup IIA (Filler at 7 Days)

Histopathological examination of this gp depicted an epithelium of the mucous membrane-side having variable thicknesses, an irregular epithelial-connective tissue interface, and occasional vacuolated epithelial cells were seen at all layers. Few keratinocytes displayed nuclear pleomorphism and hyperchromatism. Areas of ruptured epithelium were also noted. Non keratinocytes (clear cells) were also detected in basal and parabasal layers. Keratin layer

was thin and detached in some areas. Lamina propria showed some lymphocytic infiltration alongside with occasionally encountered foreign body giant multinuclear cells (Figure 1: c, d). In deeper layers, the injected HA was well-retained and bordered by lymphocytic infiltration at its periphery. Some blood vessels were dilated. Extracellular edema was noted in several areas. The skin-side of this gp displayed epithelium with numerous cell vacuolations. The CT showed areas of extracellular edema, lymphocytic infiltration as well as hyalinization of collagen fibers (Figure 1: d).

Subgroup IIB (Filler at 14 Days)

H&E-stained sections (of both mucous membrane and skin sides) of this gp showed almost the same histological features as the previous one, with a slight apparent increase in the epithelial cell vacuolations, some increase in the lymphocytic infiltrations in the CT. Dilated blood vessels engorged with RBCs were commonly seen. Areas of extracellular edema and collagen hyalinizations were also evident. Some of the injected HA was retained within the CT (Figures 1 :e-g).

Subgroup IIC (Filler at 28 Days):

Histopathological assessment of this gp's H&E-stained sections revealed an apparent reduction in the epithelial thickness of both the mucous membrane and skin-sides in several areas. Also, thinning and even loss of the keratin layer were evident in some regions. Marked apparent increase in vacuolated epithelial cells all over the epithelium was noted. The sub-epithelial CT displayed an observable increase in lymphocytic infiltration. Blood vessels were rarely spotted, some of which had small diameters and occluded lumina, while a few were dilated with collapsed lumen. Extracellular edema was detected around the perimeter of the residues of injected HA, which were still detected within the tissue. Apparently, broader areas of hyalinized collagen fibers were spotted around the injected HA (**Figures 1 h,i)**.

Alcian Blue Results

I. Control Groups (IA, IB &IC)

Histological examination of the Alcian blue-stained sections of these gps revealed normal architecture of labial tissues with absence of any pools of alcianophilic material within the CT **(Figures 2: a, b).**

Experimental Groups

Subgroups IIA, B &C

These gps showed lakes of basophilic alcianophilic material (HA filler) within the CT of both mucous membrane and skin sides of the lip. The HA appeared contracted toward the center of the pool leaving behind some strands of material **(Figures 2: c-h)**.

Picro Sirious Red Results (For Collagen Evaluation)

I. Control Groups (IA, IB & IC)

Histological study of these gp's PSR-stained sections revealed an abundance of thick bright to dark red-colored collagen fibers within the CT, denoting intact collagen bundles (Figures 3: a, b).

II. Experimental Groups (IIA, IIB & IIC)

Histological examination of these gp's PSR-stained sections displayed varying degrees of collagen type I degradation, which appeared as pink, faintly-stained fibers mainly around the injected HA . Some areas showed hyalinization and even total loss of collagen fibers. While the rest of CT (away from HA) showed intensely-stained dark red collagen fiber bundles (Figure 3: c- g).

Anti-Cox-2 Immuno-Histochemical

Control Groups (IA, IB & IC)

Immuno-histochemical examination of these gp's Anti-Cox2-stained sections displayed some immune-positive cells within the CT, mainly close to blood vessels (Figures 4 a, b).

Experimental Groups (IIA, IIB & IIC)

Immuno-histochemical examination of these gp's Anti-Cox-2-stained sections manifested marked increase in immune-positive cells, especially around pools of the injected HA. The number of immune-positive cells increased with elapsing time as it was greatest in subgroup IIC **(Figure 4: c- h).**

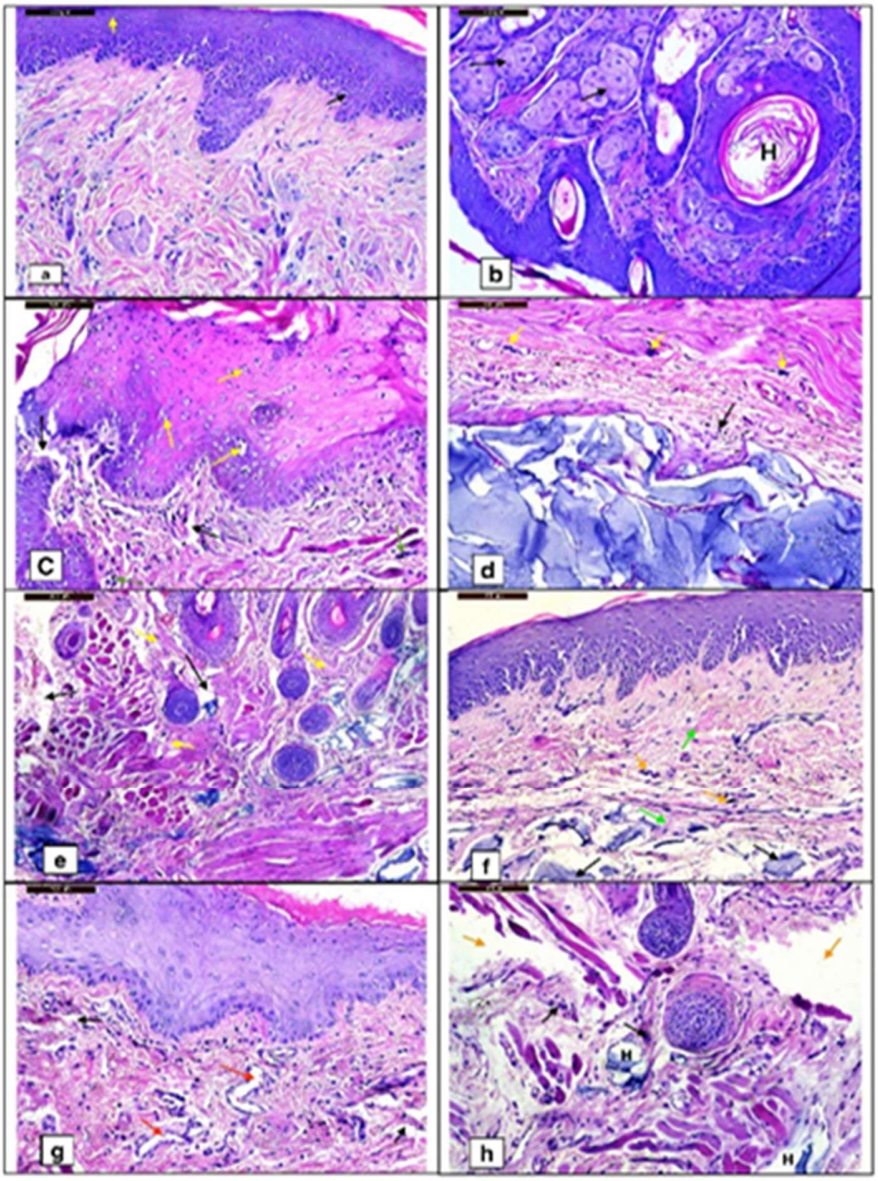

Figure 1: A photomicrograph of a rat's lip showing: **a,** mucous membrane-side of *(Subgroup IA)* in which few vacuolated cells (yellow arrows) were detected as well as few clear cells or non keratinocytes (black arrow) at basal and parabasal levels, CT showed well-developed collagen fiber bundles , numerous blood vessels of variable sizes and few inflammatory cells. **b**, skin-side of *(Subgroup IA)* well-developed hair follicles (black arrows), sebaceous glands (yellow arrows). **c,** mucous membrane-side of *(Subgroup IIA)* numerous vacuolated epithelial cells in all layers, some of which displayed nuclear changes (yellow arrows), areas of extracellular edema (black arrows), numerous inflammatory cells as well as foreign body giant cells (green arrows). **d,** mucous

membrane-side of *(Subgroup IIA)* well-retained areas of the injected hyaluronic acid filler surrounded by chronic inflammatory cell infiltration (black arrows), few multinucleated foreign body giant cells were noted (yellow arrows). **e,** skin-side of *(Subgroup IIB)* showing remnants if the injected filler around hair follicles with areas of collagen hyalinization (yellow arrows) and extracellular edema (black arrows). **f,** mucous membrane-side of *(Subgroup IIB)* residues of the filler (black arrows), wider areas of extracellular edema, marked inflammatory cell infiltration (orange arrows), dilated collapsed blood vessels and wide areas of collagen hyalinization (green arrows). **g,** mucous membrane-side of *(Subgroup IIC)* epithelium of variable thickness and numerous vauolated cells allover the epithelial layers and some clear cells basally. Areas of extrcellular edema (black arrows), marked inflammatory cell infiltration, hyalinized collagen fibrils as well as dilated collapsed blood vessels (red arrows). **h,** skin-side of *(Subgroup IIC)* massive areas of extracellular edema (orange arrows), inflammatory cell infiltration as well as multi-nuclear giant cells (black arrows) around residues of the filler (H). H&E: original mg. x 200.

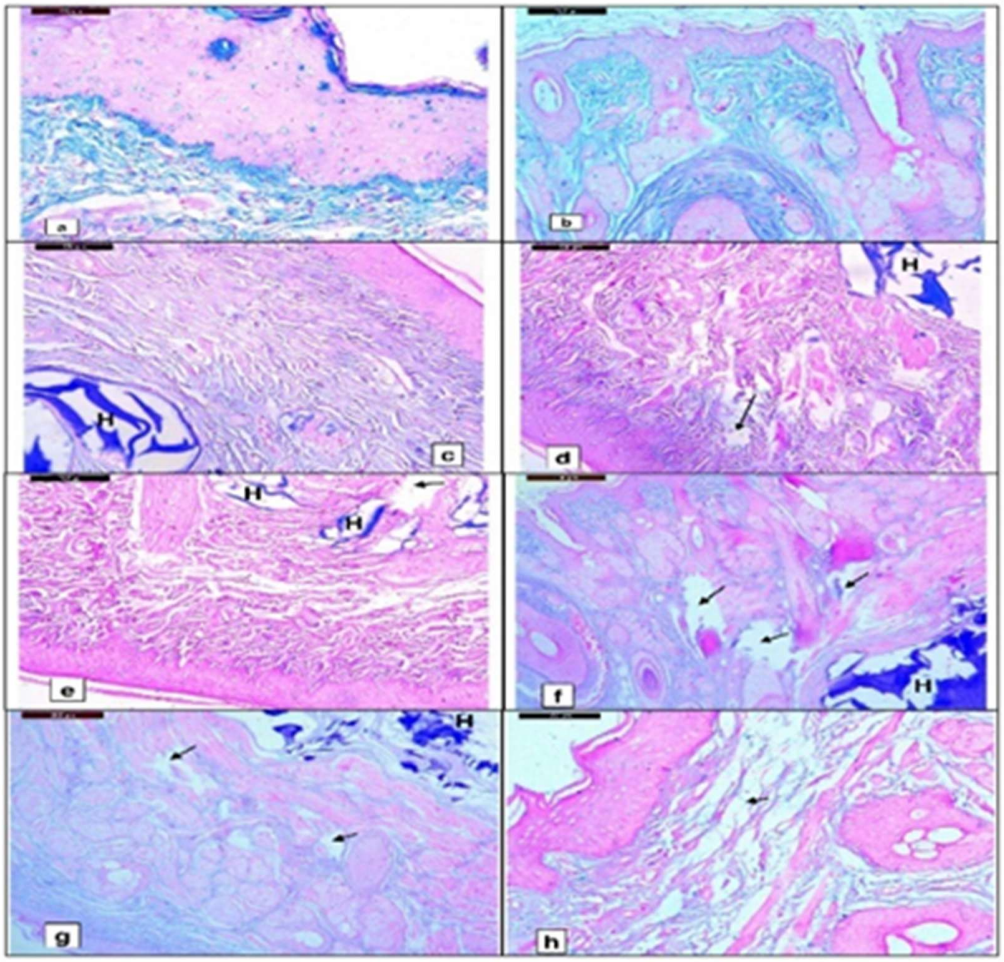

Figure 2: A photomicrograph of rat's lip showing: **a,** mucous membrane-side of *(Subgroup IA)* with normal architecture and no filler lakes detected. **b,** skin-side of *(Subgroup IA)* absence of any filler material. **c,** mucous membrane-side & **d,** skin-side of *(Subgroup IIA)* also displayed filler pools (H) and areas of extracellular edema (black arrows). **e,** mucous membrane-side of *(Subgroup IIB)* alcianophilic basophilic lakes of the filler were detected (H). **f,** skin-side of *(Subgroup IIB)* well circumscribed alcianophilic basophilic material of the filler (H) and wider areas of extracellular edema (black arrows). **g,** mucous membrane-side of *(Subgroup IIC)* wide areas of shrunken alcianophilic filler material (H). **h**, skin-side of *(Subgroup IIC)* with minimal residues of the basophilic filler material (H) and wider areas of extracellular edema (black arrows). **Alcian Blue: original mg. x 200.**

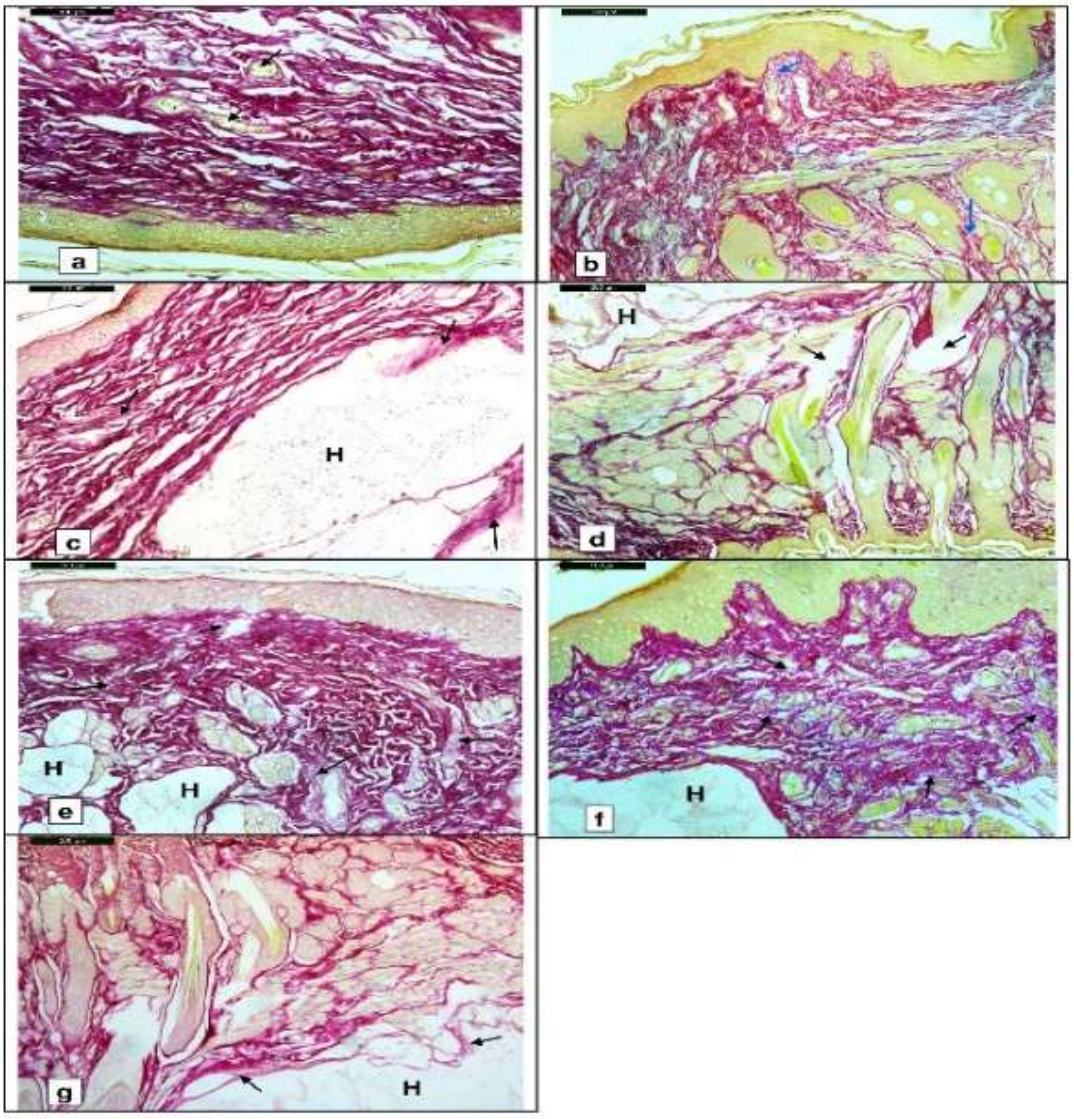

Figure 3: A photomicrograph of rat's lip showing: **a,** mucous membrane-side of *(Subgroup IA)* thick bundles of intensely stained dark red collagen fibers and well-developed blood vessels (black arrows). **b,** skin-side of *(Subgroup IA)* numerous thick dark red collagen fibers and small areas of fine faintly stained pinkish red collagen fibrils (blue arrows). **c,** mucous membrane-side of *(Subgroup IIA)* few areas of pinkish red faint collagen fibrils (black arrows) especially around the filler (H). **d,** skin-side of *(Subgroup IIA)* numerous thin faintly stained pink collagen fibrils in many areas especially around the extrcellular edema (black arrows) and in the vicinity of the injected filler (H). **e,** mucous membrane-side of *(Subgroup IIB)* showing pink collagen fibers

(black arrows) around the filler (HA). **f,** mucous membrane of *(Subgroup IIC)* small lakes of the filler (H) around which thin faintly stained pink collagen fibrils (black arrows) were detected. **g,** skin-side of *(Subgroup IIC)* a pool of the filler (H) around which wider areas of collagen fibers appeared thin and faintly stained (black arrows). **Picro Sirious Red: Original mg. x 200.**

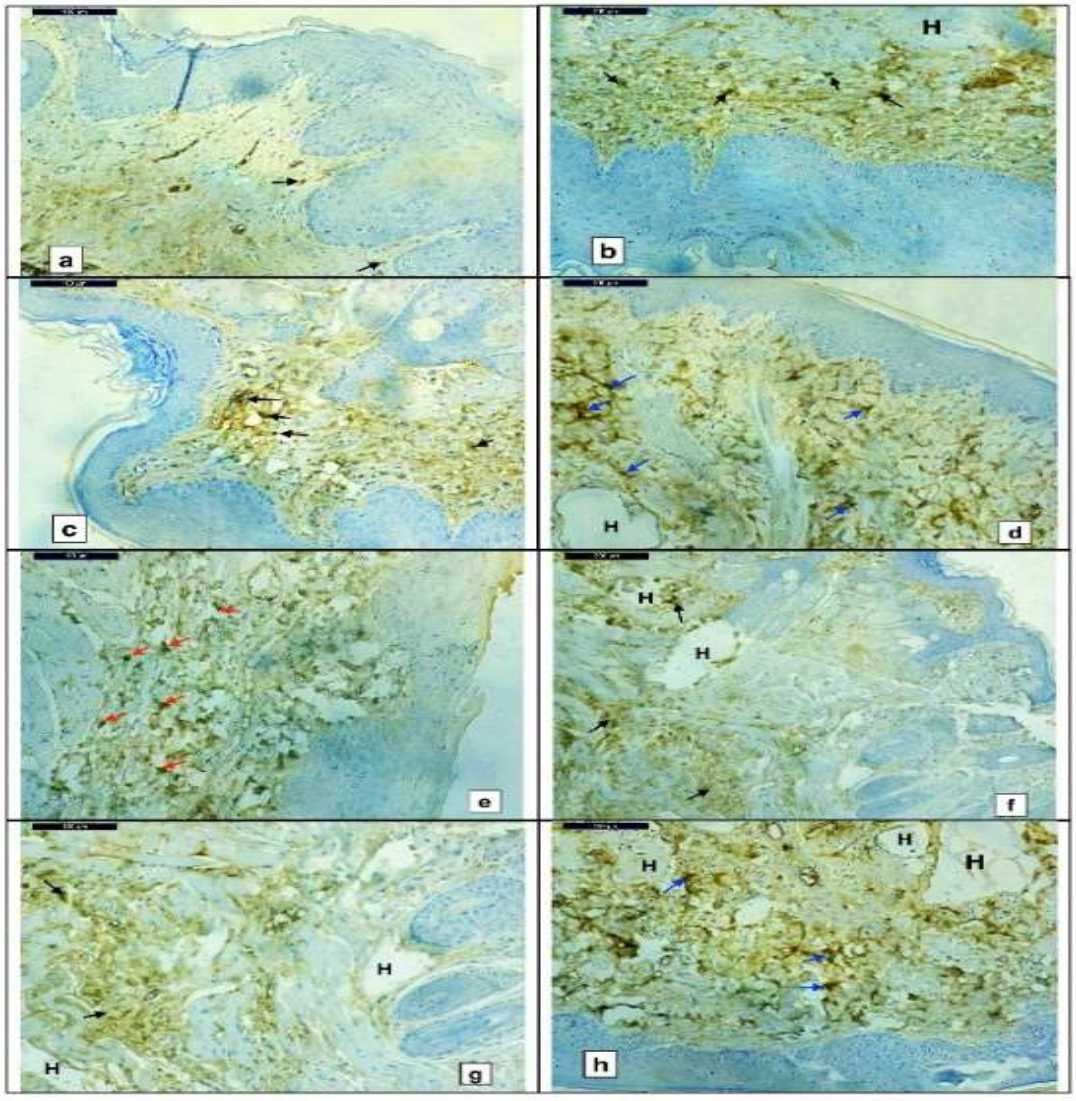

Figure 4: A photomicrograph of rat's lip showing: **a,** mucous membrane-side of *(Subgroup IA)*, with few brown colored Cox-2-positive cells in CT (black arrows). **b,** mucous membrane-side of *(Subgroup IIA)* more immune-positive cells were detected in CT (black arrows). **c,** skin-side of *(Subgroup IIA)* with obvious presence of immune-positive cells (black arrows). **d,** *(Subgroup IIB)* with many immune-positive cells (blue arrows) and pools of the filler (H). **e,** *(Subgroup IIB)* with numerous positive cells in the

CT (red arrows). f&g, skin-side of *(Subgroup IIC)* numerous positive cells (black arrows) and pools of filler (HA). h, mucous membrane-side of *(Subgroup IIC)* numerous positive cells (blue arrows) and pools of filler (H). Anti Cox-2: original mg. x 200.

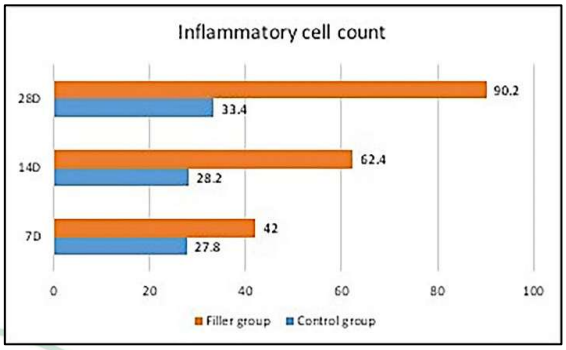

Figure 5: Column chart showing mean count of inflammatory cells in all groups.

Statistical Results

Inflammatory Cell Score

The greatest mean of inflammatory cell count was recorded in the filler group (after 28 days), whereas the lowest value was recorded in control group (after 7 days). One-way analysis of variance (ANOVA) test revealed that the difference between the intervals of the filler group was statistically significant (P<0.0001). Tukey's post hoc test revealed a significant difference between each 2 groups. (Table1, Fig 5.). Unpaired T-test revealed a significant difference between the filler and control groups (after 28 days) (P<0.0001) (Table,1).

Table 1: counting of inflammatory cells in all groups and significance of the difference using ANOVA test and unpaired T-test.

	7D	14D	28D	ANOVA P-value
Control group	27.8±8.78	28.2±2.6	33.4±6.1	
Filler group	42±3.16 ^{c}	62.4±2.7 ^{b}	90.2±18^{a}	0.000047*
T-test P-value			0.000079*	

***significant at p<0.05 Tukey's post hoc test: means sharing the same superscript letter are not significantly different.**

Cox-2-Positive Cell Score

The greatest mean of Cox-2 score was recorded in the filler group (after 28 days), whereas the lowest value was recorded in control group (after 14 days). ANOVA test revealed that the difference between the intervals of the filler group was statistically significant (P<0.0001). Tukey's Post Hoc revealed a significant difference between each 2 groups. (Table2, Fig 6). Unpaired T-test revealed a significant difference between the filler and control groups (after 28 days) (P<0.0001) (Table 2).

Table 2: Cox-2 score in all groups and significance of the difference using ANOVA test and unpaired T-test.

	7D	14D	28D	ANOVA P-value
Control group	43±6.6	22±1.6	25.4±5.1	
Filler group	85±15^{c}	110±5.16^{b}	164±11.8^{a}	< 0.00001*
T-test P-value			< 0.00001*	

***significant at p<0.05 Tukey's post hoc test: means sharing the same superscript letter are not significantly different.**

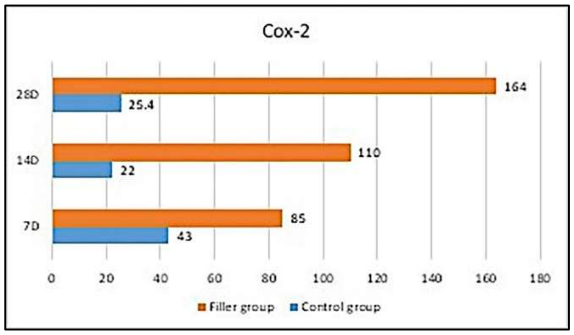

Figure 6: Column chart showing Cox-2 score in all groups.

Discussion

As time elapses, our bodies undergo structural changes (aging) that involve almost all of our tissues.Error! Reference source not found. These age-related changes occur due to accumulation of deleterious substances, mostly reactive oxygen species (ROS), within the tissues. Moreover, as we age, our bodies' natural anti-oxidant mechanisms deteriorate gradually, and hence can't cope with the increasing amounts of ROS accumulating in the tissues.Error! Reference source not found. CT changes comprise reduced vascularity, collagen degradation due to activation of tissue Matrix Metalloproteinases (MMPs) as a result of ROS accumulation.Error! Reference source not found. These structural changes are reflected externally in the form of enhanced wrinkles and volume loss, especially evident in the facial area. Rejuvenating senile tissues is more difficult to achieve than preventive anti-aging, and non-invasive procedures such as dermal fillers. HA dermal fillers have been recently introduced and gained huge popularity, although their safety and efficiency need more support by scientific researches. That's why it was appealing for us to use HA in our study. Albino rats were chosen in the current study due to the great resemblance between the structure of their labial tissues and those of the humans. Error! Reference source not found.

During histological examination of the experimental groups' H&E-stained sections, we noticed apparent increase in the vacuolated epithelial cells with elapsing time as it was more observable in the 28-day subgroupIIC. Moreover, some keratinocytes displayed nuclear changes as hyperchromatism and pleomorphism, which together with the intracellular vacuolations are signs of enhanced apoptosis, according to Saraste & Pulkki .**Error! Reference source not found.** Apoptotic changes occur naturally within our tissues but in a regulated pattern, however, it can be enhanced by the accumulation ROS within the tissue.Error! Reference source not found., Error! Reference source not found. Upon histological examination of the H&E-stained sections in the current study, we noticed statistically significant increase in inflammatory cell infiltration within the experimental groups' CT, which was time-dependent as it was greatest in subgroup IIC. Meanwhile, our immunohistochemical results revealed significant increase in the Anti-Cox-2-positive cells within subgroup IIC specimens more than other experimental or control groups. Both findings confirmed the occurrence of a foreign body reaction, which fortunately wasn't severe enough to inflict granuloma formation as reported in literature in few cases.Error! Reference source not found. The increased inflammatory cells infiltration and Cox-2 expression in our results came in agreement with Nasreen et al**Error! Reference source not found.**, who reported the presence of marked inflammatory cell infiltration together with foreign-body giant cells in response to HA dermal filler injection. In our study, the presence of variable areas of extracellular edema within the CT, which was greatest in subgroup IIC, could be attributed to the inflammatory condition. Moreover, hyalinization of collagen fiber bundles together with collagen degradation were evident in the experimental groups especially subgroup IIC. This was confirmed in our study by PSR special histochemical stain, in which many of the

collagen fibers of subgroup IIC were faintly-stained, very fine pinkish fibrils, unlike collagen fibers in control groups were it appeared thick intensely-stained dark red fibers. We attributed these findings to the local inflammatory state, which results in elevated ROS production, together with the elevated levels of pro-inflammatory cytokines (released by macrophages and inflammatory cell infiltration in the region) which in turn results in enhanced MMPs activation. The resultant MMPs activation had led to widespread collagen degradation, manifested as areas of hyalinized collagen and even progressed into areas of extracellular edema, as reported in many researches.**Error! Reference source not found.. Error! Reference source not found.** Histological examination of the experimental groups' H&E-stained sections revealed apparent reduction in the vascularity in areas at the vicinity of HA filler. Moreover, in subgroup IIC many of the blood vessels displayed narrow or even occluded lumina, while few of them depicted wide collapsed lumina, which came in accordance endothelial dysfunction, manifested as loss of vascular tone.

Conclusion

HA, although being accepted widely by many researchers, yet it induced a considerable foreign body reaction in our study.

Declarations

Funding

This study did not receive any external funding.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

The experiment was conducted according to the "Guide for the Care and Use of Laboratory Animals" 8th ed., 2011,The Research Ethics Committee of the Faculty of Dentistry at 6th of October University gave their approval for the experimental design (Approval number: RECO6U/17-2023).

Conflict of Interest

Authors declare no conflict of interest.

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