The Comparative Dermal Stimulation Potential of Constant-Volume and Constant-Amount Diluted Calcium Hydroxylapatite Injections Versus the Concentrated Form

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INTRODUCTION Biostimulation properties of diluted and hyperdiluted calcium hydroxylapatite (CaHA) injections have become increasingly popular. However, the existing data are insufficient to certify a particular dose–response pattern.

OBJECTIVE To assess and compare the dermal stimulation potentials of different concentrations of CaHA injections.

MATERIALS AND METHODS Two independent experiments (Experiment-1: constant injection volume vs Experiment-2: constant CaHA amount) included 4 study groups each, and these experimental groups were placed consecutively on the abdominal skin of a juvenile Yorkshire pig. Histopathological and immunohistochemical stainings were performed on punch biopsy materials collected 4 months after the injection day.

RESULTS The fibroblast count significantly decreased upon dilution from 1:3 to 1:19 in experiment 1 ($p = .000$) but still higher than the control group. In experiment 1, the collagen density of the concentrated form was more elevated than the 1:19 dilution and the negative control groups ($p = .034$ and .000, respectively) but similar to the 1:3 dilution ($p = .123$). No significant difference was observed between the groups regarding collagen density with a standard amount of CaHA (0.2 mL, 30%) ($p > .05$).

CONCLUSION Despite the efficacy being more pronounced till 1:3 dilution, hyperdiluted CaHA at any dilution ratio up to 1:19 can provide a higher fibroblast count than the negative control group.

The demand for minimally invasive, "lunchtime, cosmetic procedures" has increased exponentially over the past decades. The target of the interventions might be the skin, the subcutaneous musculoaponeurotic system, the fat pads, or the muscles. Over the past decade, clinicians have administered a holistic approach combining different aesthetic and cosmetic procedures. As an example, clinicians frequently combine mesotherapy with soft tissue fillers. The rationale for this preference is not only targeting different layers of the face but also improved quality and thickness of the overlying skin would avoid the need for volume enhancement in certain facial regions.[1,2]

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The efforts to produce the ideal filler material are still ongoing. Different soft tissue fillers have their advantages and drawbacks. Besides, rejuvenating mesotherapy products involve vitamins, enzymes, hormones, non–cross-linked hyaluronic acid, and natural plant extracts. Due to the absence of in-depth, evidence-based scientific data, the validity of mesotherapy regimens is still under investigation. The results were disparate in several studies admixing the clinical and histological evaluation results of mesotherapy regimens. Some participants had a visible difference without a remarkable histological change. However, mesotherapy applications are gaining increasing attention with a relatively high financial cost to patients.[3]

Several publications attributed collagen biostimulation potential to hyaluronic acid related to a mechanical effect within the dermis. This mechanical effect is uniform for all soft tissue fillers, and the tension within the dermis is hypothesized to stimulate the activity of dermal fibroblasts.[4] In addition, among different dermal fillers, a unique feature of calcium hydroxylapatite (CaHA) is its property to serve as a scaffold for regeneration.[4]

Calcium hydroxyl apatite fillers facilitate biostimulation through angiogenesis, elastogenesis, and collagen production. Furthermore, the more superficial the injection plane is maintained, the more stimulative property is pronounced. However, commercially available concentrated CaHA fillers (30%) will likely cause visible nodules and plaques,

especially in mobile regions and thin skin. Thus, FDA approved using CaHA fillers only on a deep plane, just over the periosteal tissue.[4]

However, clinicians frequently perform off-label uses of hyperdiluted CaHA fillers with remarkable treatment outcomes.[5] Recently, Corduff and colleagues[6] implemented hyperdiluted CaHA in their regimen as a "subcutaneous biostimulatory wash" even in the tear-trough region.

Because the evidence in the literature to support this practice is currently limited, this porcine study aimed to evaluate and compare the fourth-month histological findings after a single injection of concentrated versus diluted (1:1) and hyperdiluted (1:2, 1:3, 1:19) CaHA fillers versus negative control.

This study aimed to evaluate the results of 2 hypotheses in 2 different experimental groups;

1. To reveal the efficacy of standard injection volume (0.2 mL) among different experimental groups, including varying amounts of CaHA versus the negative control.
2. To evaluate the efficacy of injecting a standard amount of CaHA (0.2 mL, 30%) admixed with varying amounts of saline versus the concentrated form.

Materials and Method

This study was conducted with the approval of the Animal Experimentation Local Ethics Committee of Health Sciences University in Ankara (protocol number: 21/02 on 28.01.2021). The experiments were performed following national and international guidelines for laboratory animal care.

Treatment Groups and Experimental Procedure

Two female juvenile Yorkshire pigs were included. The animals were maintained under anesthesia during tattooing and injections. According to the descriptions of England and colleagues[7], the animals were marked permanently with Indian ink in the corners of a 4 cm^2 grid over the entire abdomen, excluding the ribs and sternum. This marking lasted throughout the study period (4 months) and allowed the identification of individual treatment square locations. All experiments were conducted on a total number of 40 squares. The adjacent squares were concurrently assigned to 4 treatment groups, repeated 10 times for each group.

During the experiments, the authors used carboxymethylcellulose gel–based CaHA filler, including 25- to 45-μm microspheres (Novuma, Burgeon Aesthetics, Ankara, Turkey, 30% wt/vol) and saline to prepare different dilutions. First, the porcine skin was shaved and cleansed with 0.5% chlorhexidine. Then, AB and PE prepared the injection materials according to the treatment groups. Finally, materials were stored at room temperature, mixed immediately before injection, and transferred to 1-mL Luer-lock syringes.

Experimental Procedure 1 (Constant Injection Volume)

All study materials were injected with a perpendicularly oriented 27-gauge, 13-mm needle to target the immediately subdermal plane. Ercan CALISKAN injected the dermal CaHA filler in its commercially available formulation to experiment 1 group 1 (E1G1) (0.2 mL 30% CaHA). Other administered treatments were a mixture of 0.05 mL 30% CaHA and 0.15 mL saline for group 2 (E1G2) (1:3 dilution) and a mixture of 0.01 mL 30% CaHA and 0.19 mL saline (1:19 dilution) for group 3 (E1G3), respectively. In addition, the authors administered an isovolumetric injection (0.2 mL) of saline to group 4 (E1G4) (Table 1).

Experimental Procedure 2 (Constant CaHA Amount)

In the second experimental procedure, the administered treatments included a standard amount (0.2 mL, %30) of dermal CaHA filler in its commercially available formulation in all experimental groups. Experiment 2 Group 1 (E2G1) received the concentrated form. Groups 2 (E2G2), 3 (E2G3), and 4 (E2G4) received the product admixed with 0.2 mL (1:1), 0.4 mL (1:2), and 0.6 mL of saline (1:3), respectively (Table 1).

Pathological Evaluation

The tissue samples were collected on the same day, determined as 4 months after the injection day. After fixation with 10% buffered formaldehyde solution, 6-μm sections were obtained from embedded into paraffin after processing. The sections were stained with hematoxylin–eosin (HE), Masson-trichrome, and Orcein stain.

TABLE 1. The Features of the Experimental Groups

	Experiment 1 (Constant Injection Volume)			Experiment 2 (Constant CaHA Amount)		
	CaHa	Saline	Final CaHa (%)	CaHa	Saline	Final CaHa (%)
Group 1 (n:10)	0.2 mL (%30)	—	%30	0.2 mL (%30)	—	%30
Group 2 (n:10)	0.05 mL (%30)	0.15 mL	%7.5	0.2 mL (%30)	0.2 mL	%15
Group 3 (n:10)	0.01 mL (%30)	0.19 mL	%1.5	0.2 mL (%30)	0.4 mL	%10
Group 4 (n:10)	—	0.2 mL	—	0.2 mL (%30)	0.6 mL	%7.5
	Total injection volume for each group: 0.2 mL			Total CaHa 0.2 mL (30%) for each group		

CaHa, calcium hydroxylapatite.

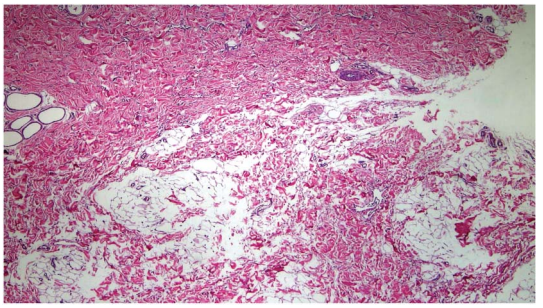

Figure 1. There is no reaction in the deep dermis (H-E, ×40).

The pathologist (M.G.) was blind to the experimental groups. Foreign body tissue reaction was assessed in 2 different sections in HE sections. Then, a semiquantitative score was found based on absent: 0, less than 1 mm (focal): 1 to 2 [total score: 1 + 0 or 1 + 1], and larger ones: 2 to 4 [total score: 2 + 0 or 2 + 2] (Figures 1,2). A hotspot in the high-power field within the region adjacent to the inflammatory reaction was determined, and fibroblasts were counted in 1 mm^2 to assess collagen content within the newly formed. Collagen density and elastic tissue ratio were also calculated for each sample by the ImageJ software program (NIH, Bethesda, MD), as previously described[8] (Figures 3,4).

Immunohistochemical Stainings

Monoclonal antibodies against elastin (bs-1756R; Bioss, Boston, MA; 1:400 dilution), collagen type I (bs-10423R; Bioss; 1:400 dilution), and collagen type III (bs-0948R; Bioss; 1:400 dilution) were performed for assessing in new collagen formation. A semiquantitative scoring was used as weak (2 points), moderate (4 points), strong (6 points), or hyperexpression (8 points), similar to the study of Yutskovskaya and Kogan[9] (Figures 5-7).

Statistical Analysis

Statistical analyses were performed using IBM SPSS for Windows, Version 22.0 (Armonk, NY). Numerical variables were shown as mean ± SD. Differences between the groups were evaluated by a 2-tailed *t*-test. A p value of $<.05$ was considered significant in all comparisons.

Ethics Approval

Gulhane Animal Experiments Ethics Committee (Project number: protocol number: 21/02 in 28.01.2021).

Results

In this experimental study, outcome measures were HE inflammation score, fibroblast count, collagen density, and the amount of elastic fibers. Table 2 depicts the numerical values of the HE inflammation score, fibroblast count, collagen density, and the amount of elastic fibers for all study groups.

Experiment 1 (Constant Injection Volume)

Hematoxylin–Eosin Inflammation Scores

CaHA was microscopically detected in all CaHA study groups. As expected, the control sites did not reveal an inflammatory pattern similar to foreign body tissue reaction. Upon intergroup comparisons, the differences between E1G1-E1G2 and E1G2-E1G3 did not reach statistical significance (p = .288 and .711, respectively).

Fibroblast Count

Fibroblasts were most concentrated within the reticular dermis. Fibroblast count did not differ between E1G1 (concentrated) and E1G2 (1:3 dilution). The fibroblast count of E1G3 (1:19 dilution) was less than E1G1 and E1G2 (p = .017 and .000, respectively); and higher than E1G4 (control group) (p = .003).

Collagen Density

Collagenesis was most pronounced within the reticular dermis. Collagen density was higher in CaHA groups when all active treatment sites were compared with the control group (E1G1+E1G2+E1G3 vs E1G4 p = .007). The collagen density of E1G1 (concentrated) was higher than E1G3 (1:19 dilution) and E1G4 (control) (p = .034 and .000, respectively). The collagen densities of E1G1 and E1G2 (1:3 dilution) were similar (p = .123).

Elastic Fiber

No significant difference was observed between the groups regarding elastogenesis ($p > .05$).

Immunohistochemical Staining

The immunohistochemical staining scores for collagen-1, collagen-3, and elastin did not differ between the experimental groups ($p > .05$).

Experiment 2 (Constant CaHA Amount)

Hematoxylin–Eosin Inflammation Scores

The HE inflammation scores did not differ comparing E2G1 (concentrated) versus E2G2 (1:1 dilution), E2G2 versus

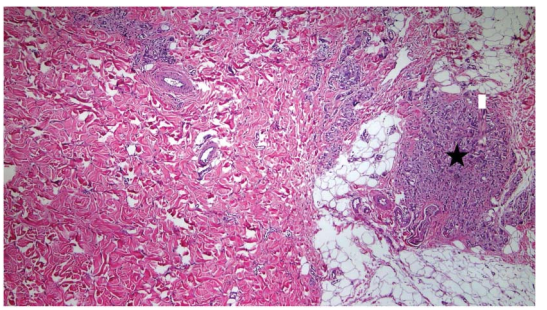

Figure 2. Focal foreign tissue reaction (*), (H-E, ×40).

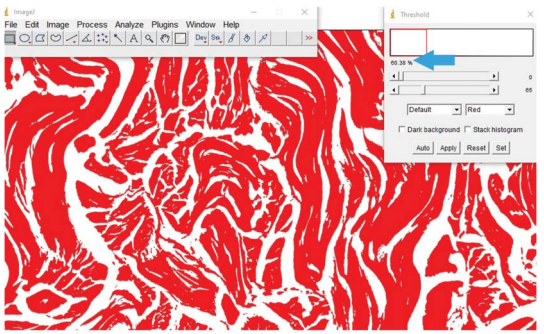

Figure 3. Measurement of collagen density as a percentage (arrow).

E2G3 (1:2 dilution), and E2G3 versus E2G4 (1:3 dilution) (p = .501, .898, and .797, respectively).

Fibroblast Count

The fibroblast count of E2G1 (concentrated) was higher than E2G2 (1:1 dilution) and E2G4 (1:4 dilution) (p = .001 and .009), but the difference between E2G1 and E2G3 (1:2 dilution) did not reach statistical significance (p = .116).

Collagen Density

No significant difference was observed between the groups regarding collagen density with a standard amount of CaHA (0.2 mL, 30%) ($p > .05$).

Elastic Fiber

No significant difference was observed between the groups regarding elastogenesis for both experimental groups ($p > .05$).

Immunohistochemical Staining

The immunohistochemical staining scores for collagen 1, collagen 3, and elastin did not differ between the experimental groups ($p > .05$).

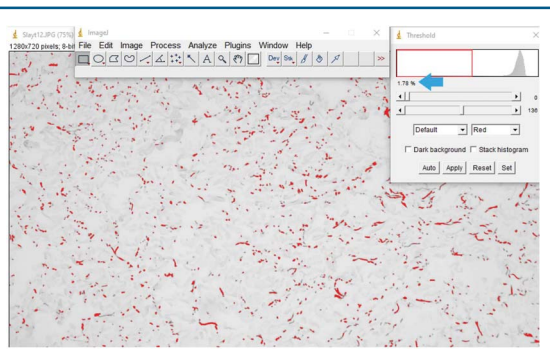

Figure 4. Measurement of elastic tissue density as a percentage.

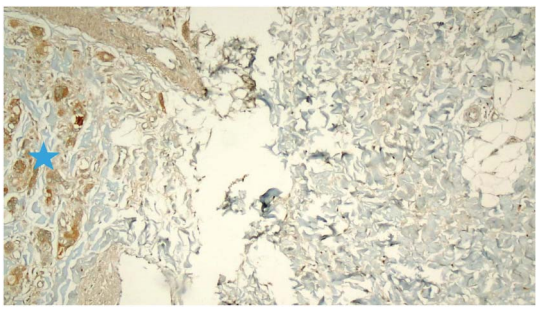

Figure 5. Foreign tissue reaction (*) and weakly extracellular immunoexpression of collagen I in perilesional tissue (×100).

Discussion

According to global consensus recommendations, CaHA formulations are considered diluted when prepared as 1:1 dilution and hyperdiluted when prepared as ≥1:2 dilution.[10,11] For the hyperdiluted form, there is no clear recommendation on the ideal ratio that can provide maximal dermal proliferation similar to the concentrated form without causing textural asymmetry and nodularity. In addition to the frequently used dilution ratios, this study included an extreme dilution ratio (1:19) group, apparently not used in clinical practice. The rationale for including this group was to test the hypothesis that serving as a scaffold, even a tiny amount of CaHA could preserve its dermal stimulation potential to a certain degree. The results confirm this hypothesis.

In this study analyzing the fourth-month results of CaHA injections through a constant volume design, the fibroblast count and collagen density of 1:3 diluted CaHA were similar to the concentrated form. Upon 1:19 dilution, both the fibroblast count and collagen density were lower than the concentrated form; however, with a higher fibroblast count compared with the negative control. The collagen density did not differ between the treatment groups through a constant product design comparing different dilutions (concentrated vs 1:1 dilution vs 1:2 dilution vs 1:3 dilution).

Calcium hydroxylapatite is a unique molecule that simultaneously provides volume replacement and biostimulant activity. The properties related to new tissue

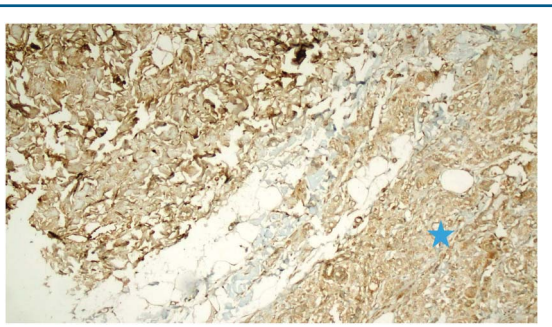

Figure 6. Foreign tissue reaction (*) and moderately extracellular immunoexpression of collagen III in perilesional tissue (×100).

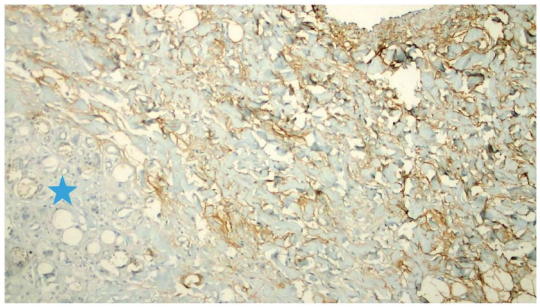

Figure 7. Foreign tissue reaction (*) and moderately extracellular immunoexpression of elastin in perilesional tissue (×100).

formation, including new collagen and elastin production and angiogenesis, begin to emerge gradually over time, increasing skin thickness and quality as well as dermal remodeling, lifting, and tightening.[11] Although the long-lasting effects of CaHA are a critically important advantage, when it is not administered by experienced, well-trained hands, asymmetry, textural changes, product visibility, nodularity, and granulomas may be inevitable.[10,12] Hyperdilution permits even distribution of microspheres over a larger surface area. The rationale for the widespread use of hyperdiluted CaHA formulations was to prevent complications, as mentioned earlier, and increase skin quality without volume augmentation.[10,13,14] Hyperdiluted CaHA should always be placed immediately below the dermis in the dermal–subdermal plane.[5]

Several hyperdilution ratios of CaHA varying between 1:2 and 1:6 have been studied in different body regions in the existing data so far. The preferences for hyperdilution mainly rely on skin thickness, as the product may be easily visible when the skin is thin. According to the experience sharing of experts, a dilution ratio of 1:2 was considered optimal for normal skin, 1:4 for thin skin, and 1:6 for atrophic skin.[9,11]

Bravo and colleagues[15] reported an 11% increase in dermal thickness at the end of the fourth month due to the combination of 1:1 diluted CaHA and hyaluronic acid in facial skin laxity. Fabi and colleagues[16] drew attention to the wrinkles in the décolleté area, which significantly improved after a single injection of CaHA (1:2 dilution). Furthermore, they emphasized the benefit of recurrent injections and reported that the improvement effect was amplified after the second injection and lasted about 1 year.

Yutskovskaya and colleagues[9] provided histologic data for the dermal stimulation potential of hyperdiluted CaHA. Initially, they shared their unpublished data comparing the dermal stimulation of 1:2 ($n = 6$) and 1:8 ($n = 7$) dilutions at the third month of injections. They determined statistically significant increases in collagen I, collagen III, and elastin without a difference between the 2 dilution ratios.[9] Depending on these preliminary results, they admixed the measurements of the 2 dilution groups and compared the results with negative control in their clinical study. The results of this study demonstrate differences from their results. The fibroblast count and collagen density decreased upon dilution from 1:3 to 1:19. Furthermore, an identical approach was maintained for collagen I and collagen III immunohistochemical stainings, surprisingly, without an apparent difference between the study groups and negative control.

The difference between immunohistochemical staining results can be attributed to several reasons. First, this study did not include preexperimental skin samples to depict the trend for dermal stimulation over a determined period, instead provided a cross-sectional analysis of the fourth-month results of the experimental groups. Second, this study used a nonhuman model, which might be a technical pitfall for the staining.

TABLE 2. HE Inflammation Score, Fibroblast Count, Collagen Density, and Elastic Fibers Amount in Experimental Groups

	Group 1 (n:10)	Group 2 (n:10)	Group 3 (n:10)	Group 4 (n:10)
Experiment 1 (standard total injection volume)				
CaHA (%)	%30	%7.5	%1.5	—
Volume	0.2 mL	0.2 mL	0.2 mL	0.2 mL
Inflammation score	2.5 ± 1.95	1.6 ± 1.7	1.9 ± 1.8	0
Fibroblast count	199.6 ± 32.9	221 ± 27.7	170.5 ± 12.3	147.9 ± 17.1
Collagen density	58 ± 2.3	55.3 ± 4.6	54.5 ± 4.2	52.0 ± 2.69
Elastic fiber	1.03 ± 0.27	0.96 ± 0.03	0.96 ± 0.04	0.97 ± 0.04
Experiment 2 (standard amount of CaHa)				
CaHA (%)	%30	%15	%10	%7.5
Volume	0.2 mL	0.4 mL	0.6 mL	0.8 mL
Inflammation score	3.3 ± 1.5	2.8 ± 1.7	2.7 ± 1.7	2.5 ± 1.7
Fibroblast count	199.8 ± 16.6	157.5 ± 27	179.3 ± 35.4	152.6 ± 30.1
Collagen density	57.5 ± 2.1	55.3 ± 4.4	55.4 ± 2.7	57.5 ± 3.6
Elastic fiber	0.97 ± 0.05	0.95 ± 0.01	0.96 ± 0.04	0.98 ± 0.06

CaHa, calcium hydroxylapatite; HE, hematoxylin–eosin.

Coleman and colleagues[17] assessed the neocollagenesis property of concentrated CaHA in a canine model. As an unexpected finding, they detected that the fourth-week collagen amount was higher than the that of 16th week, increasing stepwise thereafter until the 58th week. They suggested that swelling and scar formation were the reasons for the higher collagen amount in week 4. In the current analysis, although the results on new collagen production confirm the literature, the absence of elastin increments compared with negative control may be associated with the relatively short follow-up period.

Several methods, even the injection procedure itself, can induce collagen production. This study adopted the identical injection method for all experimental groups, including the negative control group. The fibroblast count measurements consistently decreased upon dilution, and fibroblast counts of all treatment groups were higher than those of the control group, indicating a class effect devoid of physical stimuli.

Fibroblast count is an earlier indicator of dermal stimulation, substantially followed by elastin and collagen production. The initial response to CaHA might be fibroblast count increment, occurring concurrently or consecutively with fibroblast activity stimulation. Thus, collagen production could be considered a late event. Two possible scenarios arise after the close-up evaluation of the 1:19 dilution group results, pointing out that fibroblast count increases without collagen density improvement compared with the control group. First, in the 1:19 dilution group, collagen enhancements might occur less obviously and slower than in the other study groups, appearing later after 4 months, and this study could not assess late results. Second, a tiny amount of CaHA might be able to increase fibroblast count but not sufficient to enhance fibroblast activity.

The major limitation of this study was that the outcomes only represented the results of a single CaHA injection at a single time point. The authors adopted a less complicated study design and determined these settings (single injection vs repeated injections, sampling at 4 months vs different sampling times) considering the ease of analysis. The authors suggest that a more extended follow-up duration and repeated injections may produce more robust collagenesis or elastogenesis. The duration of the obtained effect is another enigma that was not in this study's scope.

Conclusion

The results of this study representing the fourth-month results of a single injection suggest that despite the efficacy being more pronounced till 1:3 dilution, hyperdiluted CaHA at any dilution ratio up to 1:19 can provide a higher fibroblast count than the negative control group.

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