

Enzymatic Recontouring of Auricular Cartilage in a Rabbit Model

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Objective: To evaluate the effectiveness of contouring auricular cartilage in a rabbit model using biologically active enzymes injected subcutaneously.

Methods: The first phase determined the most effective volume and concentration required to affect the cartilage. To accomplish this task, we used *ex vivo* rabbit ears from a slaughterhouse. In the second phase, we injected 1 mL of hyaluronidase (150 U per milliliter of isotonic sodium chloride solution [saline]), elastase (1 mg per milliliter of saline), or saline into the ears of live rabbits. The study took place at the Madigan Army Medical Center (Tacoma, Wash), and included 10 animals. In each rabbit, we injected the test compound in one ear and saline in the other ear (control). We injected hyaluronidase in 5 ears and elastase in 5 ears. After injection, the ears were contoured and splinted for 4 weeks. In the third phase, we changed the injection pathway in 5 animals.

Results: At 4 weeks, 4 (80%) of the 5 ears injected with hyaluronidase showed full response and 1 (20%) had a partial response. Of the 5 ears injected with elastase, 4 (80%) showed a full response while 1 (20%) demonstrated a partial response. There was a response in all 10 of the ears injected with a test compound. Of the 10 control ears, 3 (30%) showed a partial response. At 6 weeks, approximately 6 (30%) of the ears had maintained contour demonstrating a full response. The difference between the test ears and the control ears was statistically significant ($P=.006$). Compared with the control ears, the results were statistically significant for elastase ($P=.004$) and hyaluronidase ($P=.02$). Overall, both agents demonstrated a subjective and objective response compared with control ears.

Conclusion: This study demonstrates that bioactive enzymes and splinting can be effective in correcting ear deformities in a rabbit model.

Arch Facial Plast Surg. 2005;7:104-110

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NONSURGICAL TREATMENT of auricular (external ear) deformities has been described in the literature dating back to the 19th century.¹ In 1891, Monks¹ reported failure in his efforts to treat prominent ears with a brass compression device in the *Boston Medical & Surgical Journal*. As a result, he developed one of the first reported surgical otoplasty procedures. Almost 100 years later, Smith² suggested that certain ear deformities were acquired rather than congenital. In 1984, Matsuo et al³ described successful non-surgical correction (with molding and taping) of congenital auricular deformities in the early neonate. Their group evaluated 1000 infants and observed that this correction was successful if undertaken within the first 3 months of life.

Auricular cartilage consists of chondrocytes (cartilage cells) and intercellular materials (eg, hyaluronic acid and proteoglycans) that are bound within a collagen and elastin matrix. Matsuo et al³ postulated that these cartilage components are more loosely bound in the neonate due to increased hyaluronic acid. Matsuo et al³ and Hung et al⁴ suggested that elevated maternal estrogens during pregnancy greatly increase the levels of hyaluronic acid in fetal cartilage, reaching maximal levels immediately after birth. Both studies showed a positive correlation between elevated maternal estrogen levels, increased fetal hyaluronic acid, and increased neonatal auricular malleability.

In this study, we sought to recreate the malleability of auricular cartilage as described by Matsuo et al³ and Hung et al⁴ by targeting hyaluronic acid and elastin in

the rabbit ear. We injected proteolytic enzymes or agents targeted to these auricular components to potentially degrade the cartilage, making it more malleable without destroying it. The enzymes or agents chosen were hyaluronidase and elastase for the reasons described above. We splinted the ears for 4 weeks with a simple ear splint formed of dental rolls and a 2-0 polypropylene suture.

The study was divided into 3 phases. The first phase quantitated the optimal amount and concentration of the injected agents in ex vivo ears obtained from a slaughterhouse. We then evaluated the subjective and histological results in the phase 1 specimens. This phase allowed evaluation of the caustic effects of the enzymes in ex vivo rabbit ears rather than using live animals. In phase 2, we injected the optimal bioactive enzymes into the ears of live rabbits. The ears were splinted for 4 weeks. We graded and photographed the results at weeks 4 and 6 (2 weeks after removal of the splints). In phase 3, we evaluated another injection pathway in 5 additional rabbits to rule out any discrepancies of cartilage molding based on the enzyme-injected pathway.

In our review of the literature, we found no studies that incorporated hyaluronidase or elastase injections for the contouring of either congenital or acquired auricular deformities. Based on the results of this study and possible future studies, there may be an adjunctive benefit of using biologically active enzymes to improve the outcome of surgical otoplasty.

METHODS

The Department of Clinical Investigation, Madigan Army Medical Center, Tacoma, Wash, approved the study protocol. This study was initiated with a pilot phase (phase 1) to evaluate the efficacy of various concentrations of hyaluronidase and elastase injected subcutaneously in the ears of ex vivo rabbits. In phase 1, we randomly assigned 27 ears from euthanized rabbits to 1 of 2 test groups: group 1, hyaluronidase and group 2, elastase. Each group of 9 ears was further divided into 3 concentration cohorts. In group 1, we injected 2 mL of hyaluronidase, 75, 150, or 225 U per milliliter of isotonic sodium chloride solution (saline), in the ears (Wyeth Laboratories, Philadelphia, Pa). In group 2, we injected 2 mL of elastase, 0.5, 1.0, or 1.5 mg per milliliter of saline, in the ears (Sigma-Aldrich, St Louis, Mo). We then sutured the ears at 180° from the vertical or erect position with a 2-0 polypropylene suture. The sutures remained in position for 5 days and were preserved by refrigeration in airtight bags.

We evaluated the auricular cartilage and soft tissue of the ears for soft-tissue reaction and necrosis at 24, 48, and 72 hours after the subcutaneous injection. The ears in phase 1 were evaluated subjectively and histologically to determine whether cartilage destruction or simple disaggregation of the auricular cartilage had occurred. Histological preparation and evaluation was performed by the Department of Pathology, Madigan Army Medical Center. The principal investigators (P.L.M. and P.E.G.) objectively and subjectively evaluated the ears' ability to retain the contoured shape. Based on the information gathered in phase 1, we concluded that 1 mL was an optimal volume of injection based on the size of the rabbit ear and the injection pathway. The optimal concentration of enzymes was determined by folding the ear (determined subjectively by the authors [P.L.M. and P.E.G.]) and by looking at the enzymatic effects on the auricular cartilage (determined histologically by



Figure 1. Simple ear splint made of 2 dental rolls and a 2-0 polypropylene suture.

the reviewing pathologist). The optimal concentrations resulting in disaggregation and not destruction of the auricular cartilage matrix were 150 U of hyaluronidase per milliliter of saline and 1.0 mg of elastase per milliliter of saline (see "Results" section).

In phase 2, we injected 1 mL of the enzyme concentrations determined to be effective in phase 1 to groups of 5 live rabbits per treatment agent and concentration. In each rabbit, we randomly assigned the ear (ie, left vs right) injected with the test compound, and the other ear was the control ear. In the control ears, we injected 1 mL of physiological saline in the same region and manner as the treatment ears. After the injections, treatment and control ears were contoured with manual manipulation and splinted with dental rolls and 2-0 polypropylene sutures (**Figure 1**). We performed all injections and manipulations after administering general anesthesia, consisting of the veterinary tranquilizer xylazine hydrochloride (5-10 mg/kg administered intramuscularly) and ketamine hydrochloride (30-45 mg/kg administered intramuscularly 10 minutes after xylazine). This regimen consistently allowed 20 to 40 minutes for required ear preparations, injections, and manipulations. All rabbits received systemic analgesia during anesthesia and for at least 24 hours after injection. We placed soft Elizabethan collars on the anesthetized rabbits to prevent the rabbits from scratching at or damaging the ear splints.

We injected the solution in a subcutaneous plane along a predetermined injection pathway parallel to the longitudinal axis of the ear. Two injection pathways were ultimately chosen. We chose the first injection pathway, a vertical direction along the medial surface of the rabbit auricle, because of the rigid consistency of the auricular cartilage in this anatomical area (**Figure 2**). This injection was administered to the initial group of 10 live rabbits in phase 2. Based on the initial results at splint removal, we selected a second injection pathway, a transverse direction across the entire surface of the auricle (**Figure 3**). In phase 3, we used the second injection pathway in 5 additional rabbits. Based on the differences in the folding patterns, we separated phase 2 and phase 3. While we may



Figure 2. First injection pathway along the convex (medial) surface, parallel to the longitudinal axis of the rabbit's ear.



Figure 3. The second injection pathway used, which followed a transverse direction across the entire surface of the rabbit's ear.

have had a larger sample size by combining the 2 phases, the results would have been altered by the track of the injection pathway affecting the auricular folding observed after splint removal.

The ear splints remained in place for 4 weeks. After the splints were removed, the ears were objectively and subjectively evaluated for angulations and resilience of contour. We developed a contour grading system, applying a 0° to 90° scale with 0° corresponding to the vertical or erect position, and used the scale to objectively measure success and maintenance of contouring (**Table 1**). After splinting, we visually assessed and photographed the gross results. The rabbits received an inhaled anesthetic while we removed the splints, recorded and documented results, and obtained histological specimens.

Histological 1.0-cm specimens of auricular cartilage obtained from phase 2 and phase 3 rabbit ears were placed in formalin. After fixation, the specimens were sectioned at 0.5-cm intervals and submitted for examination. A representative 4- μ m section of each specimen was stained with hematoxylin-eosin and Verhoeff stains. Two blinded pathologists reviewed the specimens. The evaluation criteria for the hematoxylin-eosin-stained specimens were presence of chondrocytes and reactive or regenerative changes in the chondrocytes. The Verhoeff-

Table 1. Grading System Used in Evaluating Results

Degree of Angulation	Maintenance of Contour	Quantitative Score
0-30	No response	1
30-60	Partial response	2
60-90	Full response	3

stained sections were evaluated for loss of elastic fibers compared with controls using the following grading system: slight (less than 10% loss of elastin fibers), mild (10%-25% loss of elastin fibers), moderate (25%-50% loss of elastin fibers), or severe (more than 50% loss of elastin fibers).

The results were subjected to statistical analysis for quantification. We used the following statistical tests: χ^2 , G^2 , contingency coefficient, and Cramer V. Groups were then compared for significance using Kruskal-Wallis and Mann-Whitney tests. Comparisons were made between the bioactive enzymes and controls and between the individual enzymes. Finally, rank comparisons were made between groups of subjects.

RESULTS

PHASE 1

Three concentrations of hyaluronidase (75, 150, and 225 U per milliliter of saline) were injected in the ex vivo rabbit ears. The 75-U concentration demonstrated no tissue edema and had no appreciable effect on the auricular cartilage. The 150-U concentration demonstrated minimal tissue edema, greater maintenance of contour after suture removal, and decreased resiliency of the auricular cartilage compared with the control ear (**Figure 4**). The 225-U concentration showed no increase in measurable effectiveness compared with the 150-U concentration. In the histological results, the 150-U concentration maintained contour without demonstrating cartilage destruction.

Three concentrations of elastase (0.5, 1.0, and 1.5 mg per milliliter of saline) were injected into the ex vivo rabbit ears. The 0.5-mg concentration had a minimal effect on the contour of the cartilage, but there was decreased cartilage resilience. The 1.0-mg concentration demonstrated greater maintenance of cartilage contour, minimal soft-tissue edema and erythema, and no cartilage destruction. The 2.0-mg concentration showed excellent maintenance of contour but also showed cartilage destruction, necrosis, and soft-tissue ulceration and granulation.

In the results with all concentrations of elastase, there was more profound soft-tissue edema and erythema compared with the hyaluronidase concentration. The most efficacious concentrations in phase 1 were 150 U of hyaluronidase per milliliter of saline and 1.0 mg of elastase per milliliter of saline.

PHASE 2

The phase 2 treatment group included 10 animals. Five rabbits received injections of 2 mL of hyaluronidase, 150

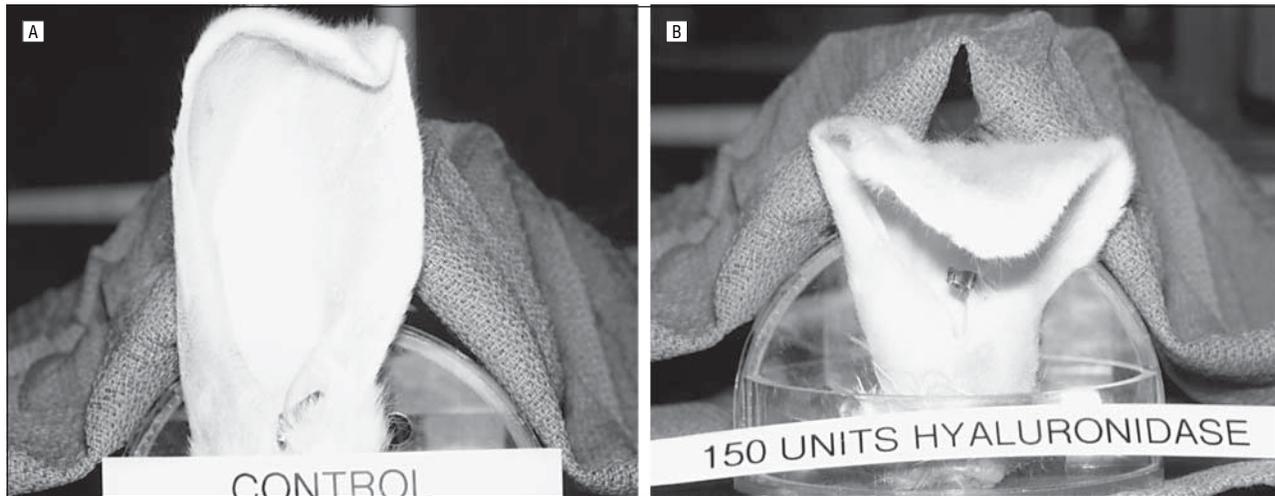


Figure 4. A, Control ex vivo rabbit ear; B, ex vivo ear demonstrating contour after treatment with hyaluronidase and splinting.

Table 2. Phase 2 Contouring Results After a 4-Week Splinting Period

Results	No. (%) of Ears			Total
	Control	Hyaluronidase	Elastase	
Full response	0 (0)	4 (80)	4 (80)	8 (40)
Partial response	3 (30)	1 (20)	1 (20)	5 (25)
No response	7 (70)	0 (0)	0 (0)	7 (35)
Total	10 (100)	5 (100)	5 (100)	20 (100)

Table 3. Phase 3 Contouring Results After a 4-Week Splinting Period

Results	No. (%) of Ears			Total
	Control	Hyaluronidase	Elastase	
Full response	0 (0)	3 (100)	1 (50)	4 (40)
Partial response	3 (60)	0 (0)	1 (50)	4 (40)
No response	2 (40)	0 (0)	0 (0)	2 (20)
Total	5 (100)	3 (100)	2 (100)	10 (100)

U per milliliter of saline, and 5 rabbits received injections of 2 mL of elastase, 1.0 mg per milliliter of saline. In all the experimental animals, the contralateral ear was injected with 2 mL of saline (control). In this phase 2 group, we used the first injection pathway, along the medial aspect, parallel to the longitudinal axis of the ear (Figure 2).

The 5 ears injected with the hyaluronidase concentration had the following results: 4 (80%) showed full response, 1 (20%) showed partial response, and 0 (0%) showed no response. The 5 ears injected with the elastase concentration had the same results: 4 (80%) demonstrated full response, 1 (20%) demonstrated partial response, and 0 (0%) had no response. Of the 10 control ears, 0 (0%) had a full response, 3 (30%) had a partial response, and 7 (70%) had no response. At 6 weeks (2 weeks after removal of the ear splints), 3 (60%) of the 5 ears injected with the elastase concentration and 1 (20%)

of the 5 ears injected with the hyaluronidase concentration had maintained contour, demonstrating a full response (Table 2).

PHASE 3

In phase 3, we explored the possibility that the injection pathway might alter the postsplinting results. The concentrations of hyaluronidase and elastase were identical to those used in the phase 2 rabbits. The ears were injected across the entire surface of the auricle (Figure 3). Phase 3 results can be reviewed in Table 3. Of the 5 treated ears, 4 (80%) showed a full response (maintained contour), 1 (20%) showed a partial response, and 0 (0%) showed no response. All 3 of the ears injected with the hyaluronidase concentration showed a full response. Of the 2 ears injected with the elastase concentration, 1 (50%) showed a full response and 1 (50%)



Figure 5. Contour obtained by changing the injection pathway to a transverse injection across the entire auricle.

showed a partial response. Of the 5 control ears in phase 3, 2 (40%) had a partial response and 3 (60%) had no response. At 6 weeks (2 weeks after splint removal), 2 (40%) showed a full response, 2 (40%) showed a partial response, and 1 (20%) showed no response. Although the number of ears is small, 4 (80%) of 5 ears showed a response at 6 weeks.

Compared with the control ears, both concentrations of hyaluronidase ($P = .02$) and elastase ($P = .004$) were effective in recontouring the rabbit ear. There was no statistically significant difference between elastase and hyaluronidase or between ears injected with protease and those that were not. However, compared with the control ears, the results in the ears treated with a test compound were significantly different ($P = .006$).

Histological specimens were obtained from 12 of the animals for evaluation by 2 blinded pathologists. Hematoxylin-eosin and Verhoeff stains were used to evaluate the specimens. The criteria selected for evaluation were the presence of chondrocytes, indicating reactive or regenerative changes in the cartilage, and the percentage of elastin fiber loss, indicating the effects of the elastase injection. It is often difficult to distinguish between benign and malignant cartilage in histological specimens. Ignarro et al⁵ observed that chondrocytes lose their nuclei when stressed or damaged. We also observed that treating cartilage with an elastase enzyme in various concentrations can reduce the number of elastin fibers that are visualized on a microscopic preparation.

COMMENT

Previous investigators³ have proposed that neonates have more malleable cartilage due to increased hyaluronic acid, which disappears with maturity. Matsuo et al³ have shown that the rigidity of auricular cartilage increases with age because of decreased levels of hyaluronic acid and increased levels of elastin. In our study of rabbits, we sought to partially alter the auricular cartilage to a less rigid, moldable state by adding degradative enzymes before splinting.

In combination with collagen, elastin has been shown to form the matrix that binds chondrocytes and intercellular materials in auricular cartilage.⁶ Degradation of the noncollagenous protein mucopolysaccharide matrix in rabbit cartilage in vitro has been reported using various enzyme preparations.⁶ In our study, we chose elastase to enzymatically break down elastin, resulting in more malleable auricular cartilage.

The rabbit provides an ideal model for this study. In their study of congenital auricular deformities, Matsuo et al³ injected estradiol benzoate in rabbits to determine its effect on cartilage elasticity. Rabbits possess abundant auricular cartilage that is readily accessible. Matsuo et al³ reported that the auricular cartilage of the rabbit is an excellent animal model for disorders of the human external ear. Matsuo et al³ have demonstrated the non-surgical correction of the neonatal auricle. Yotsuyanagi et al⁷ performed an experimental adult trial with less predictable outcomes than those reported by Matsuo et al.³ To our knowledge, previous studies have not used enzyme injections to improve contouring results. The average splinting time in the previous study by Matsuo et al³ was 4 to 6 weeks, and they reported a slight loss of the result immediately after splinting. As shown in the results of our study, hyaluronidase or elastase use may improve the postsplinting results after a 4-week splinting time.

The splinting results were photographed and graded using the scales shown in Table 1. The ears were observed immediately after splint removal and again after 5 minutes of inspection and manipulation. **Figure 5** shows a photograph of a rabbit ear treated with the elastase concentration and splinted for 4 weeks. The ear maintained its molded contour after splint removal. All the ears injected with a test solution demonstrated a response. At 6 weeks (2 weeks after splint removal), 60% had retained their contour, demonstrating a complete response. In the control ears, no ears demonstrated a response. Thus, the 30% with a partial response at 4 weeks decreased to 0% at 6 weeks. The results for the 3 groups at 6 weeks are shown in Table 3. The results of our study show that using enzyme injections with splinting is more effective than splinting alone.

There was a statistically significant difference between the test group and the control group ($P = .006$). Using the Mann-Whitney test, we found that the difference between the elastase group and the control group was also statistically significant ($P = .004$). Compared with the control group, the results in the hyaluronidase group were different ($P = .02$). Overall, both agents demonstrated a subjective and objective response compared with the control group. Elastase was the only agent that showed statistically significant results ($P = .004$) using the Mann-Whitney test. There was little to no soft-tissue reaction to hyaluronidase. Although the results were statistically significant, the elastase group showed a more profound soft-tissue reaction consisting of erythema, edema, granulation, and auricular cartilage cell loss. The results of this study validate the assumption that elastase is the most effective injected enzyme. Some of the control ears demonstrated a partial response from the splinting alone, but this response was short-lived. None of the control ears demonstrated a response at 6 weeks.

While evaluating the phase 2 results observed with both agents, it seemed possible that the injection pathway might have influenced the results. An additional treatment group was used to evaluate this possibility. The phase 3 group received the same volume and concentration as the first animals, but the agent was injected in a transverse pattern across the entire auricle. Similar results were observed. Overall, 80% showed a full response, 20% showed a partial response, and 0% showed no response. All the ears injected with hyaluronidase demonstrated a full response. Half the ears injected with elastase demonstrated a full response, and half showed a partial response. There was clearly a subjective difference in the results between the injection pathways, as observed in **Figure 6**, but it was not statistically significant. The difference in contour can be clearly seen when comparing Figure 4A and B. The number of test subjects in this additional group was small ($n=5$), but overall 80% showed a response at 6 weeks (2 weeks after splint removal).

The number of control ears demonstrating a response due to splinting alone did not surprise us. Since the auricular cartilage is malleable and the ear can be held in a defined shape, the ears may retain their shape after splint removal. This shape was short-lived and could not be seen within 72 hours of splint removal. None of the control ears showed a full response from splinting alone. Furthermore, our statistical analysis shows that the results of the addition of the bioactive enzymes were significant compared with the control ears ($P=.006$).

In this study, the hyaluronidase concentration demonstrated small numbers of chondrocyte cell loss compared with the control cartilage. The hyaluronidase concentration did not cause a generalized soft-tissue reaction or alter elastin fiber density. The elastase concentration demonstrated a more profound loss of chondrocyte cells and elastin fiber density at the site of injection compared with the surrounding tissues and with control ears. We saw a more pronounced soft-tissue reaction with the elastase injections; however, no necrosis was observed using the 1.0-mg concentration. In all specimens examined histologically, only one of the ears injected with elastase demonstrated a small granuloma. In our preliminary *ex vivo* studies using higher concentrations of elastase (2.0 mg), we observed cartilage and soft-tissue necrosis. We believe that the histological evidence obtained in this study validates our proposal that the bioactive enzymes are effective in remodeling or remodeling the rabbit auricular cartilage without destroying it. The auricular chondrocytes do demonstrate stress in the form of cell nuclei loss and changes in the concentration of elastin fiber, but cartilage destruction was not observed.

The results demonstrate that bioactive enzyme injections using hyaluronidase and elastase with cartilage contouring can be an effective nonsurgical treatment for auricular abnormalities. The information obtained in phase 1 using *ex vivo* rabbit ears prevented additional animal use and distress. This was a pilot study at our institution to explore a method of testing pharmaceutical preparations in *ex vivo* subjects, thereby avoiding additional

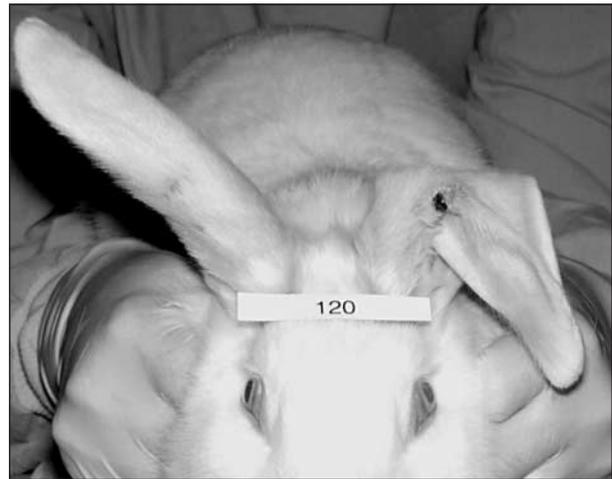


Figure 6. Rabbit ear demonstrating maintenance of molded contour after injection of elastase and splint removal.

distress and destruction of animals. The results were invaluable for determining the optimal volume of concentration of test agents to be used in our live subjects. The live rabbits in phases 2 and 3 tolerated all the surgical procedures, including splint application and removal, with minimal anesthesia and sedation. They also tolerated the auricular splints for 4 weeks without a great deal of distress. The final volume and concentration obtained in phase 1 proved to be statistically effective in our phase 2 animals.

These results offer promise of nonsurgical manipulations with bioactive enzymes to correct auricular deformities. The elastase concentration was the most effective injected compound but elicited a more pronounced inflammatory reaction compared with the hyaluronidase concentration and the control. Additional studies will be required with elastase before it should be used in humans. Long-term contouring results are still in question. Approximately 60% of the ears maintained their contour 2 weeks after splint removal. The results of phase 3 demonstrated slightly better results and slightly improved maintenance of contour 2 weeks after splint removal. The injection pathway subjectively appeared to affect the observed results, but the difference was not statistically significant. It would be difficult to draw any formal conclusions about the phase 3 group because of the small number of subjects.

It is possible that longer splinting periods or repeated injections during the splinting period would improve long-term results. Statistical objective data demonstrate that the injections were better at maintaining contour compared with the controls. Histological objective data demonstrate auricular cartilage remodeling without destruction. It is not likely that injections of bioactive enzymes will alleviate the need for otoplasty in the future. Although effective in the neonatal period, auricular splints are not as easily tolerated or effective later in life. Furthermore, additional studies are required to fully evaluate the effect of the bioactive agents in a human model. It may prove to be an important alternative treatment to surgical otoplasty or as an addi-

tive, synergistic therapy to improve surgical otoplasty results in the future.

CONCLUSIONS

This study demonstrates that nonsurgical management can be effective in correcting ear deformities using bioactive enzymes and splinting in a rabbit model. Hyaluronidase and elastase are effective agents compared with saline. The results obtained in this study reached statistical significance. Obvious limitations of this study include the possible errors inherent in working with an animal model and the small sample size in phase 3. The other question is whether these enzymatic injections will be effective in humans. We believe that the results can be applied in humans. We agree with the authors of other studies that rabbit ear cartilage is an excellent model and closely relates to human auricular cartilage. Thus, we are confident that enzymatic injections will have a place in ear cartilage recontouring. Future studies will be required to augment the results of this study.

Accepted for Publication: November 16, 2004.

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