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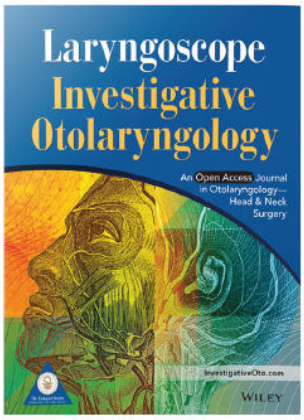

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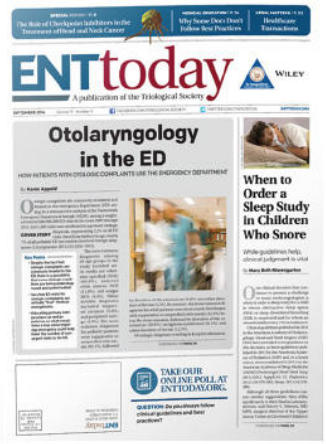

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Decreased Intramuscular Calcium Hydroxyapatite Implant Resorption in a Murine Model of Osteoporosis

Derrick R. Randall; Nogah Nativ-Zeltzer; Daniel J. Cates; Steve P. Tinling; Peter C. Belafsky

Objective: Calcium hydroxyapatite (CaHA) is a common material for vocal fold injection augmentation. Durability is variable, and factors involved in implant longevity are not understood. Animal models of osteoporosis show decreased bone density and increased mineral liberation, suggesting CaHA retention may be altered in these conditions.

Study Design: Prospective murine investigation.

Methods: Fourteen skeletally mature, 10-month-old female Sprague-Dewley rats were treated by one of three interventions: oophorectomy, laparotomy without oophorectomy (sham), or monthly risedronate injection (90 μg/kg, subcutaneous). CaHA was implanted into the right lateral thigh muscle in all animals at the time of procedure or first risedronate injection. After 17 weeks, all rats were sacrificed, and the residual CaHA isolated from excised lateral thigh muscle through incubation in a 900 °C calcinator for 9 hours.

Results: Mean CaHA mass remaining in the oophorectomy group was 65.9 (standard deviation ± 16.1) mg, compared to 44.4 ± 10.0 mg CaHA in the risedronate group and 48.6 ± 7.5 mg in the sham group. One-way analysis of variance found a statistically significant difference between the oophorectomy and risedronate groups but not between the sham and other groups, $F(2,11) = 4.404$, $P = 0.039$.

Conclusion: Persistent estrogen deficiency in a murine model of osteoporosis demonstrated decreased rate of CaHA resorption. This suggests that hormone alterations associated with osteoporosis may alter the longevity of CaHA implant resorption through an uncertain mechanism.

Key Words: Vocal fold augmentation, voice, laryngology, vocal fold paralysis, calcium hydroxyapatite.

Level of Evidence: NA.

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INTRODUCTION

Unilateral vocal fold paralysis (UVFP) is a clinical entity frequently encountered in otolaryngology–head and neck surgery, with the most common etiologies attributed to iatrogenic injury, neoplasm, and idiopathic sources.[1] Various symptoms can occur, most typically weak, breathy, diplophonic voice; excessive strain, pain, and vocal fatigue; aspiration, coughing with eating, and impaired cough; and shortness of breath with exertion or prolonged conversation. The main interventions for UVFP involve increasing bulk to the affected vocal fold, thereby improving approximation of the leading edges of the vocal folds to obtain better glottic closure. This is primarily achieved through static vocal fold augmentation techniques such as thyroplasty or material injection. Numerous vocal fold injection materials have been developed and used under different indications that take into consideration UVFP etiology, expected duration, and tissue pliability.

One of the most common injection laryngoplasty materials used for longer-term medialization effect is micronized calcium hydroxyapatite (CaHA). CaHA injections improve vocal quality, vocal fold closure pattern, respiratory parameters, and patient voice satisfaction.[2,3] The duration of effect typically varies from 8 to 36 months, but reasons for this variation remain unknown.[4] In some instances, effect duration is brief and attributed to insufficient material injection that appears once gel carrier is resorbed. In some cases, however, the decreased duration extends beyond the expected 3- to 4-month time period associated with gel carrier resorption, which suggests that early CaHA loss may be a factor.[4]

CaHA resorption occurs through activation of the reticuloendothelial system via tissue histiocytes or macrophages, which develop from hematopoietic stem cells and are analogous to osteoclasts responsible for bone resorption and remodeling. CaHA microspheres are slowly resorbed by tissue macrophages, with some ingrowth of collagen fibrils between microspheres.[5–7] Studies reporting CaHA durability and duration of effect in laryngology and cosmetic surgery used human and animal subjects by measuring stroboscopic appearance along with subjective

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voice outcomes,[2,4,8] histologic analysis,[5,6,8] and radiographic measures.[9,10] Although these methods identified anatomic and histologic features of CaHA resorption, they are not able to accurately report the quantity of material lost over time. Materials bioengineering studies aiming to purify CaHA from bone with no residual organic component identified several techniques using heat or chemical dissolution of organic tissue, which should be applicable to the measure of residual intramuscular CaHA.[11–13]

Osteoporosis forms a significant burden of disease among the population affected by vocal fold immobility and glottic incompetence. At present, no scientific literature describes the rate of CaHA resorption in populations affected by osteoporosis. Recognition of altered CaHA resorption due to osteoporosis may alter therapeutic approach among patients undergoing medialization procedures or adjuvant medication to preserve the functional effect derived from CaHA injections. A well-established animal model of osteoporosis is ovariectomized female rats (OVX), which creates an estrogen-deficient postmenopausal metabolism.[14,15] This model develops decreased bone mineral density and strength relative to healthy controls, consistent with osteoporosis, and is the FDA-approved preclinical model for osteoporosis research.[16,17]

In this study, we analyzed differences in the residual amounts of CaHA remaining from intramuscular injections in murine models of osteoporosis, sham surgery controls, and bisphosphonate (BP) treatment. We achieved accurate measure of residual CaHA by high-temperature calcination to isolate purified CaHA 17 weeks after intramuscular CaHA injection into rat thigh muscle.

MATERIALS AND METHODS

Study Population

Fourteen skeletally mature, 10-month-old female Sprague-Dewley rats. This study was reviewed and approved by the University of California, Davis, Institutional Animal Care and Use Committee, protocol 19099.

Surgical Procedure, Risedronate Injection, and Calcium Hydroxyapatite Implantation Techniques

All animals were fasted overnight prior to surgical procedures. Animals were individually anesthetized with 2% halothane (Sigma-Aldrich, St. Louis, MO) in 2 L/minute (min) oxygen in an anesthetic induction chamber until asleep. Animals were then transferred to a sterile surgical field in a supine position and kept sedated with a nose cone inhalational device providing 2% halothane at in 2 L/min oxygen. Animals were monitored pre-, intra- and postoperatively according to institutional guidelines.

Oophorectomies were performed by standard protocol. After adequate anesthetization, the animal was placed in the supine position on a sterile field, shaved, and prepped with betadine to the inguinal lines. Each ovary was removed via separate, sequential laparotomy incisions through the inguinal crease and underlying muscle and abdominal fascia. The periovarian fat was grasped and pulled out through the incision to identify the ovary and attachment to the uterine horn, which was ligated with a 2-O silk suture (Ethicon, Somerville, NJ) and removed sharply. The laparotomy incisions were closed with running 4-0 polyglactin 910 sutures (Ethicon). Sham surgeries were performed through identical procedures, but the ovarian fat and ovary were not identified or removed.

Monthly risedronate (Sigma-Aldrich) injections were administered to rats in the control group at weeks 0, 4, 8, and 12. Risedronate (90 μg/kg) was injected subcutaneously in the left thigh under anesthetic for the first injection and then without anesthesia thereafter.

CaHA (Prolaryn Plus, Merz, Franksville, WI) implantations were completed immediately after the oophorectomies, sham procedure, or initial BP injection. Injections were performed on anesthetized rats in the prone position. The location and depth of the right lateral thigh muscles were palpated, and then 0.2 mL of CaHA was injected into the muscle through a 22-gauge needle.

Calcium Hydroxyapatite Isolation

After 17 weeks, all rats were sacrificed in a CO2 chamber. A small number of rats underwent a postmortem computed tomography (CT) (Xoran Technologies, Ann Arbor, MI) using 0.4-mm slice thickness with coronal and sagittal reformatting to localize the CaHA. To excise the CaHA with certainty that no residual material was left and no bone was harvested, the right lateral thigh muscles were exposed by making a parasagittal skin incision over the caudal spine and hip joint, and then a skin flap was sharply elevated until the entire lateral thigh was exposed. The CaHA location was confirmed by visual inspection and palpation, and then the entire lateral thigh musculature was sharply excised from the bone and cartilage by severing the tendinous attachments to the bones of the leg and pelvis. The excised lateral thigh muscles were immediately placed in individual preweighed ceramic trays. The residual CaHA was isolated through incubation in a 900 °C calcinator for 9 hours to remove all organic tissue. The crucibles and remaining CaHA were then cooled to room temperature. Ceramic trays and their CaHA content were weighed on a Sartorius microbalance (Thermo Fisher Scientific, Waltham, MA) to determine the mass of residual CaHA by differential weight.

To validate our surgical and isolation techniques, an initial pilot test was run using one OVX animal that was sacrificed 1 month after surgery and injection but not included in analysis. This was conducted on muscle injected with CaHA and harvested after sacrifice, muscle harvested away from CaHA implant site but injected with CaHA ex vivo after sacrifice (positive control 1), CaHa without tissue (positive control 2), and muscle harvested away from CaHA (negative control).

RESULTS

All test animals survived the perioperative period and reached the planned date of sacrifice. Fourteen rats were included in this study, with one rat excluded from the OVX group. This animal had wound dehiscence that required revision incision closure and we were unable to access the site of CaHA injection. Figure 1A shows a representative axial CT scan with CaHA dispersed through the lateral thigh muscle, which highlights the difficulty of accurate volumetric analysis in irregular shapes by imaging techniques. The CaHA is seen deep to the skin and investing fascia, embedded within the muscle. This is confirmed on gross dissection (Fig. 1B). No CaHA could be identified outside of the muscle.

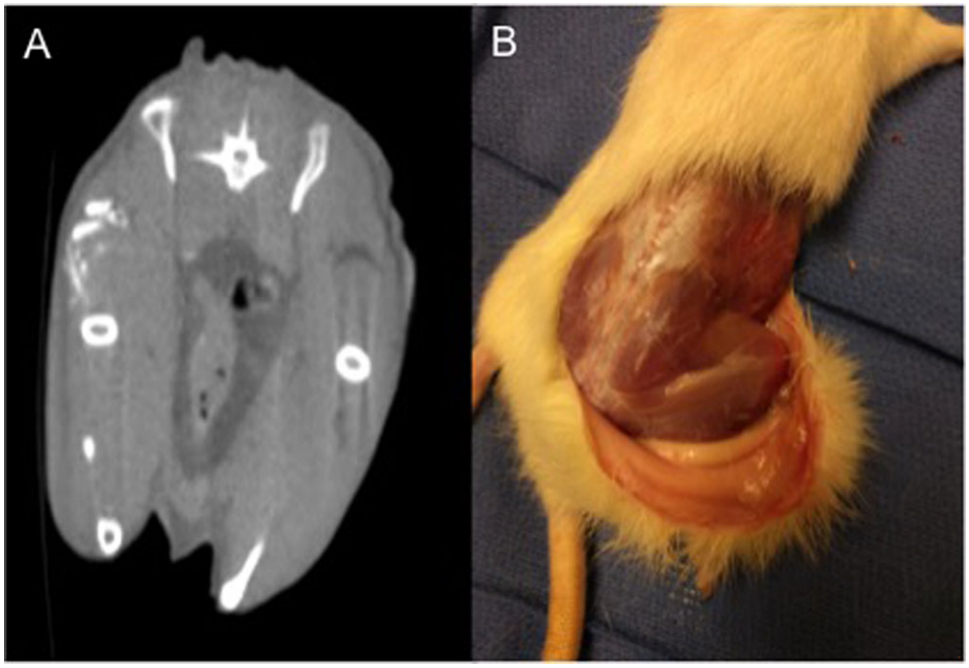

Fig. 1. Representative axial computed tomography scan (1A) and surgical dissection of lateral thigh muscle (1B) in an animal following sacrifice 4 months post-CaHA implantation. CaHA can be identified within the thigh musculature but not extruding from the muscle. CaHA = calcium hydroxyapatite. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

Our methodology validation confirmed complete muscle ablation in the negative control with CaHA preservation in the positive controls and test model (Fig. 2). No significant residue was produced in the negative control that contained only muscle, indicating that no formation of CaHA from tendon or soft tissue and no inadvertent bone harvesting occurred.

Table I shows the mean rat weights and residual CaHA for each group. Mean CaHA mass remaining in the oophorectomy group was 65.9 (SD ± 16.1) mg, compared to 44.4 ± 10.0 mg CaHA in the risedronate group and 48.6 ± 7.5 mg in the sham group, respectively. One-way analysis of variance found a statistically significant difference between groups, $F(2,11) = 4.404$, $P = 0.039$. Scheffe post hoc testing identified statistically significant difference between the oophorectomy and risedronate groups ($P = 0.048$) but not between the sham and other groups (Fig. 3).

DISCUSSION

In this article, we present intriguing results of reduced CaHA resorption following intramuscular

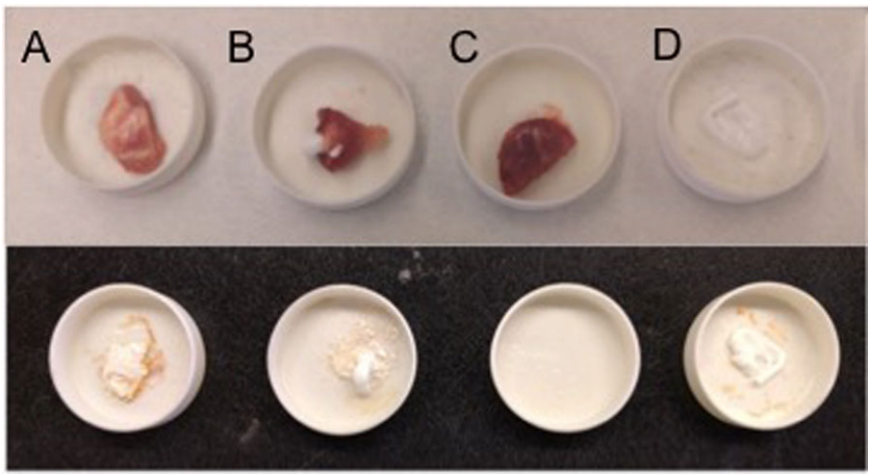

Fig. 2. Pre- and postcalcination crucibles demonstrating calcination efficiency and accuracy in isolating CaHA from harvested tissue specimens compared to control samples. (A) Muscle injected with CaHA and harvested following 17 weeks (test). (B) Muscle harvested away from CaHA implant site but injected with CaHA ex vivo after sacrifice (positive control 1). (C) Muscle harvested away from CaHA (negative control). (D) Unmodified CaHA without tissue (positive control 2). CaHA = calcium hydroxyapatite. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

TABLE I.
Mean Rat Weights and Residual CaHA Identified

Group	n	Age at Sacrifice (months)	Mean Animal Weight (g ± SD)	Residual CaHA Mass (mg ± SD)
OVX	4	14	346 ± 18	65.9 ± 16.1
Sham	5	14	318 ± 25	48.6 ± 7.5
Risedronate	5	14	340 ± 24	44.4 ± 10.0

CaHA = calcium hydroxyapatite; OVX = ovariectomized; SD = standard deviation.

injection in a murine model of osteoporosis compared to animals that received monthly BP injections. This outcome was unexpected and contradictory to our working hypothesis that osteoporosis would increase the rate of CaHA resorption through calcium liberation and that BP therapy would prevent loss. This has implications for the use of CaHA as a vocal fold augmentation material in patients using BPs and raises questions about the mechanisms of CaHA resorption in muscle.

We assume that CaHA resorption follows a similar mechanism to bone remodeling through local cellular acid dissolution, which may not be true. It seems possible that an important mechanism involved may the implantation of CaHA in laryngeal muscle with no surrounding bone, which is a physiologically different environment than the well-studied bone and implant literature. Animal models of bone loss injected intraosseously with micronized CaHA show increased bone development, suggesting CaHA uptake for utilization in local bone formation.[18] This is in line with our initial hypothesis that OVX rats would resorb CaHA faster to fortify bone strength, but the local environment of intramuscular injection may prevent effective CaHA mobilization. Although osteoclasts, histiocytes, and macrophages are all mesenchymal stem cell derivatives of the hematopoietic system, they are ultimately different cells with unique physiologic purposes and cell surface signatures. In bone, osteoclasts are

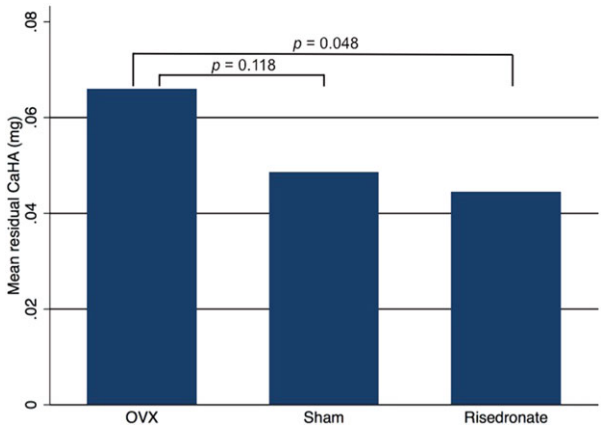

Fig. 3. Residual CaHA content for OVX, sham surgery, and risedronate groups. Analysis of variance with post hoc Scheffe testing identified a statistically significant difference in residual CaHA mass between the OVX and risedronate groups. CaHA = calcium hydroxyapatite; OVX = ovariectomized. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

recruited to the site of action by local production of M-CSF and RANKL, possibly also by local hypoxia, and mediated by vitronectin receptor.[19] There, CaHA is dissolved within the organic scaffold by acid dissolution in lysosomes produced by osteoclasts. In bone, both CaHA and collagen are reabsorbed by osteoclasts in pits or trenches, which can be regulated at different rates based on local tissue environment.[20] Osteoclasts are not involved in CaHA resorption from the larynx, and the regulation therein may be different. Instead, intramuscular and subdermal CaHA microspheres are slowly resorbed by tissue macrophages, with some ingrowth of collagen fibrils between microspheres.[5–7] Histologic studies of intramuscular and subdermal CaHA found that type I collagen converts to type III collagen, as with normal tissue healing, which leads to residual soft tissue after CaHA removal to provide tissue bulk.[7,10,21]

Estrogen is a well-known factor in disease states, and through dysregulation of the reticuloendothelial (monocyte–macrophage) system it is reported to be involved in progression of osteoporosis, autoimmune disease, atherosclerosis, and neurodegeneration, among others.[22] The changes that occur in the estrogen-mediated cellular function of bone resorption and remodeling in osteoporosis continues to be discovered. A systematic review of fracture healing in the OVX model noted delayed fracture healing and decreased biophysical strength.[23] Several studies investigate risk of osseointegration failure in estrogen deficiency through osteoporotic patients or animal models of osteoporosis, summarized in a recent systematic review finding higher implant failure rate in estrogen deficiency.[24] Yamazaki et al. observed that CaHA-coated titanium implants placed in OVX model rats produced equivalent volume of cortical bone in contact with the implant compared to sham surgery rats, but cancellous bone production was less in the OVX group, indicating local differences in bone remodeling and deposition.[25] Either BP or estrogen therapy appear able to reduce the bone loss in OVX rats fitted with titanium implants.[26–28]

We question whether use of BP may successfully sequester CaHA within bone but preferentially allow calcium liberation from the intramuscular CaHA to maintain the deranged calcium homeostasis in our OVX model. BPs are a cornerstone osteoporosis therapy to reduce fracture risk by preventing loss of bone strength. Their bone mass preservation effect is achieved by inhibiting osteoclast bone resorption through binding to calcium crystals.[29,30] The driving factors for BP mechanism of action are inhibitory effect on osteoclasts and affinity for bone material and adsorption to bone.[31] BPs are adsorbed to exposed CaHA and then get taken up by osteoclasts.[31] X-ray crystallographic and in vitro studies identified BPs are competitive inhibitors of the enzyme farnesyl pyrophosphate synthase and prevent posttranslational modification of small GTP -binding proteins.[32–34] One may expect BPs to have equal affinity for CaHA regardless of tissue location, but BPs bind preferentially to bone with high turnover rates and thus are not evenly distributed throughout the body.[35] This combined with a mechanism independent of osteoclasts may explain why the sham and BP group had similar resorption profiles.

This study is a unique analysis of CaHA resorption but has several limitations. First, it is a small study that needs replication in a larger series. The low power is apparent in the absence of difference between the OVX group and the sham group, although sham surgery and risedronate outcomes were similar. Second, it would be useful to follow a cohort of each study arm for longer periods of time so that we could be certain that the gel carrier has completely resorbed and provided access to CaHA for physiologic resorption as well as BP adsorption; however, the protocol did not allow for enough animals to do this. Therefore, we selected a time frame that should have the gel carrier eliminated. Furthermore, our method of isolation makes the presence of gel carrier irrelevant because this will completely burn off during calcination. Third, our OVX model, although well established as a good model of osteoporosis, has advanced skeletal age. The rat skeleton is mature at 12 weeks, so we selected 10 months of age to perform the oophorectomies to be certain that bone development was complete and would closely mimic human condition in typical osteoporosis and age. Finally, our BP study group was receiving medication but was not estrogen-deficient. Although this should not affect the risedronate effect on CaHA resorption, it is not typically used in the setting of normal estrogen production. This may provide a confounder, but we wanted to be certain any changes in the risedronate arm was due to medication and not a secondary condition.

CONCLUSION

Our study utilizes a murine model of osteoporosis to evaluate the effect of estrogen deficiency and osteoporosis on intramuscular CaHA resorption in comparison to sham surgery controls and BP therapy. This found the estrogen deficient animals experienced less CaHA resorption than the risedronate group and likely less than the sham surgery controls. This finding has implications for the use of CaHA in patients with osteoporosis and those taking BP therapy and warrants further investigation.

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