Xenotransplantation of Microencapsulated Parathyroid Cells as a Potential Treatment for Autoimmune-Related Hypoparathyroidism

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Abstract

Objectives: **Hypoparathyroidism occurs as a part of a complex autoimmune syndrome or iatrogenically after neck surgery. The disease presents many challenges, such as hypocalcemia, hyperphosphatemia, and low/undetectable parathormone levels. Allotransplantation of parathyroid tissue or cells has been reported as a promising option to overcome these effects. Transplantation of microencapsulated parathyroid tissue or cells offers an immune escape, which particularly restores the parathyroid function for autoimmune-related hypoparathyroidism. So far, clinical and in vivo studies have demonstrated limited graft survival and instability for the available biocompatible materials. In addition, the transplant site, proper local isolation, and biocompatibility of materials are directly related to survival rate.**

Materials and Methods: **A microencapsulated parathyroid xenotransplant model by using high guluronic acid-containing ultrapure alginate transplanted into rat omentum was tested in vivo for 1 year.**

Results: **After stability of empty microcapsules was ensured, parathyroid cells were microencapsulated and transplanted in rats, with results compared versus rats with naked (nonencapsulated) parathyroid cells (both groups followed for 64 weeks). Rats remained normocalcemic, and preinflammatory cytokine levels showed dramatic changes. Despite a delay posttransplant, parathormone levels increased significantly. All retrieved microencapsules elicited pericapsular fibrotic overgrowth; however, the fibrosis area was shown to be well tolerated.**

Conclusions: **The possible role of accumulation/cell infiltration of immune response remains to be elucidated. In conjunction with this, the use of nonencapsulated parathyroid cells was also positively correlated with survival rates. A similar evaluation using ultrapure alginate materials and omental transplantation may enable the future determination for the long-term effects of correction of parathormone insufficiency in patients with severe hypocalcemic responses and other endocrine diseases.**

Key words: *Hypocalcemia, Microencapsulation, Parathyroid allotransplantation, Parathormone insufficiency.*

Introduction

The most common causes of hypoparathyroidism occur as a complication during thyroidectomy, and this form may become permanent. Another cause of hypoparathyroidism can be a part of complex autoimmune syndrome.[1,2] The disease itself can be accompanied by hypocalcemia, hyperphosphatemia, and low or undetectable parathormone (PTH) levels.[3] People who are diagnosed with this condition experience numbness, tingling, cramping around feet and fingers, bone pain, and fatique, as well as have severely reduced quality of life. Patients with this condition must use calcium, active vitamin D analogs, and antiphosphorus supplementation throughout their life.[4]

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**Y. E. Ersoy and E. Aysan contributed equally to this work.*

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Currently, parathyroid allotransplantation (PTX) is the only curative treatment that increases the level of PTH[5] and is an acceptable approach for patients with hypoparathyroidism.[4,5] Another therapy option is recombinant drugs, but these are expensive and are only suitable for a limited number of patients.[6]

Previously, several microencapsulated PTX techniques have been described, but 2 major concerns always remain: (1) the source material selection (eg, cell or tissue[7]) and (2) determination of the optimal biocompatible transplant material. Each approach comes with positive and negative outcomes. As already known, the main problem for transplantation is immune rejection; however, microencapsulation has been seen as a superior applicable method.[8-10] Microencapsulation may be performed with many different materials. Among natural biocompatible materials, alginate is particularly preferred.[11,12] Herein, we microencapsulated parathyroid cells (Ptc) by using α-L-guluronic acid-rich ultrapure alginate and performed xenotransplantation into rat omentum. Our aims were to demonstrate the stability of microencapsules and to evaluate the proinflammatory responses and graft survival.

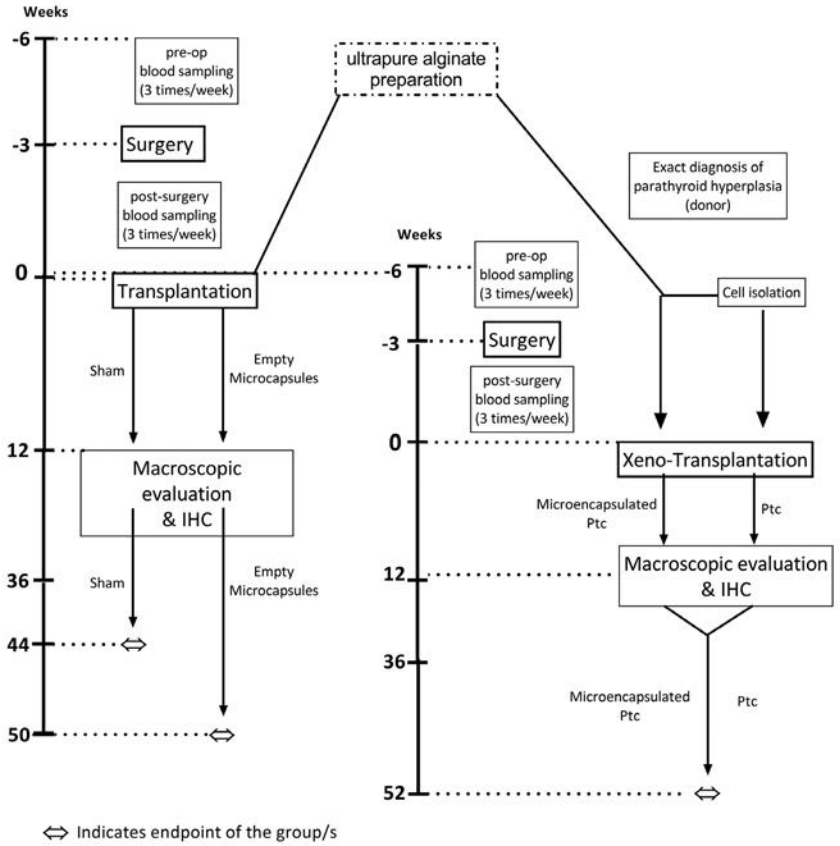
Materials and Methods

The animal study was approved by the Local Experimental Animals Ethics Committee (approval number: 2015/204). Xenograft preparation was performed after approval was received from the Local Human Ethics Committee (approval number: 71306642-050.01.04-19444). All protocols were in accordance with the ethical guidelines of the Helsinki Declaration, and written informed consent was obtained from the human donor. Methods and synopsis of the study are illustrated in Figure 1.

Animal experiments and parathyroidectomy procedure

Experiments were carried out on 32 female Wistar albino rats (12-15 weeks old) with an average weight of 240 g. Rats were housed and fed ad libitum and randomly divided into the following groups: empty

Figure 1. Methods and Synopsis of the Study



Abbreviations: IHC, immunohistochemistry; pre-op, preoperative; Ptc, parathyroid cells

microcapsules (n = 10), Ptc transplanted (n = 10), microencapsulated Ptc transplanted (n = 10), and sham (n = 2) (isotonic saline solution-injected group after laparotomy). Blood sampling was made from the jugular vein according to the methods of Parasuraman and colleagues.[13] Parathyroidectomy was performed on the 32 rats under sterile conditions.

Encapsulation of empty microcapsules

Ultrapure alginate was purchased from NovaMatrix. Low-viscosity (20-200 mPas), low-endotoxin (≤100 EU/g), and high guluronic acid content (≥60%) sodium alginate (Pronova UP LVG, NovaMatrix, Oslo, Norway) was prepared with ultrapure water with a 2% ratio (wt/vol), which was sterilized by ultraviolet exposure for 15 minutes as described previously.[14] Gelled alginate (0.5 mL) was mixed with isotonic saline solution at a 28% ratio. Capsulation was developed by calcium being used at a 300 mosM $CaCl_2$ solution and followed with a washing step for removal of the remaining $CaCl_2$. For the empty microcapsule group, isotonic saline solution (1 mL) containing empty microcapsules was transplanted into the omentum of each animal (n = 10).

Preparation of xenograft and microencapsulation

One male patient (32 years old) who had parathyroid hyperplasia due to chronic renal failure was enrolled into the present study. Half of each of the resected glands was delivered to the pathology laboratory for an exact diagnosis. The remaining parts of the glands were snap-frozen and stored at −80 °C; samples underwent the rapid thaw method before use. Parathyroid cell isolation and viability determination were assessed as previously described.[15] Before the transplant process, Ptc were cultivated with McCoy's 5A (modified) medium and placed in an incubator at 37 °C with 5% CO_2 in a humidified atmosphere. After overnight cultivation, cells were suspended in isotonic saline solution, and then viability was assessed with a Muse Cell Analyzer (Merck Millipore). For the group that underwent transplant with Ptc only, isotonic saline solution containing 50×10^6 Ptc were transplanted into the omentum of each animal (n = 10). Afterward, the remaining cell pellets were resuspended in 2% ultrapure alginate (preparation as described above).

Ultrapure alginate (0.5 mL, 2%) was mixed with a final volume of 150 μL isotonic saline solution that contained 50×10^6 Ptc in suspension prepared for each animal in the microencapsulated Ptc transplant group (n = 10). The alginate-cell suspension was placed into a 30-gauge needle and dropped into 300 mosM $CaCl_2$ solution. Spheroids were rapidly formed upon contact with the $CaCl_2$ solution. The resulting microencapsules were counted and collected into tubes containing 1 mL of isotonic saline solution.

Transplantation into the omentum

All transplant procedures were performed under sterile conditions for each experimental group (microencapsulated Ptc transplant group, empty microcapsule transplant group, and nonencapsulated Ptc transplant group). For animals receiving microencapsulated Ptc, approximately 28 to 33 microencapsulated Ptcs were transplanted into each animal's omentum. At week 12 and 52, rats in the microencapsulated Ptc and empty microcapsule transplant groups underwent omentectomy for histopathological evaluation. Other organs were controlled macroscopically, and removal was performed only when adherence of microcapsules occurred to other organs (except omentum). Only the Ptc transplant group was evaluated macroscopically.

Sandwich enzyme-linked immunosorbent assay

The following protocol was adjusted and optimized based on the work from Aydin.[16] The following solutions were used: coating buffer (0.01 M sodium carbonate buffer, pH 9.6), blocking buffer (1% [wt/vol] bovine serum albumin in 1× phosphate-buffered saline [PBS] with 0.05% Tween 20), 1× PBS (pH 7.4), washing buffer of PBS-T (PBS containing 0.05% [vol/vol] Tween 20), antibody dilution buffer (PBS containing 1% [wt/vol] bovine serum albumin and 0.05% [vol/vol) Tween 20), 2 M hydrogen peroxide as a stop solution (at 1:1 vol), and substrate solution. The substrate solution was prepared by combining 2 mL of solution/microplate of 1 mg 3,3′,5,5′-tetramethyl benzidine (TMB) in dimethyl sulfoxide with 9 mL of 0.1 M citrate phosphate buffer (pH 5.0) containing 1 μL of 30% hydrogen peroxide. All chemicals were purchased from Merck except TMB (from Sigma). All primary and secondary antibodies were purchased from Abcam.

Anti-rat PTH polyclonal antibody was used as a coating and a detection antibody. Colorimetric detection was performed with anti-rat immunoglobulin G antibody. Anti-human PTH monoclonal antibody was used as a coating antibody. Anti-

human PTH antibody of human PTH (hu-PTH) was used as a detection antibody; for colorimetric detection, anti-rabbit immunoglobulin G antibody was used. Standard preparation was performed with recombinant hu-PTH protein with a 1 to 500 pg/mL dilution range.

For the PTH sandwich enzyme-linked immunosorbent assay (ELISA), undetachable, high-binding 96-well microplates (Nest Biotechnology) were coated with antibody, which was diluted in a coating buffer (100 μL/well) and subsequently incubated at 4 °C overnight (up to 18 h). Wells were then aspirated and washed 2 times with PBS-T (250 μL/well) and blocked with blocking buffer (200 μL/well) for 1 hour at room temperature. After the blocking step, wells were aspirated and 100 μL of serially diluted standards and serum samples were added in duplicate. Microplates were incubated at 4 °C overnight (up to 12 h) and then placed at room temperature for further processing (around 10-20 min). Samples were aspirated and washed 3 times with PBS-T (250 μL/well). Detection antibody (100 μL) was added to each well and incubated for 2 hours at room temperature, which was then followed with aspiration and washing steps. Afterward, horseradish peroxidase-labeled colorimetric detection antibody (100 μL) was added to the wells, and microplates were incubated for another 1 hour at room temperature. As a final step, microplates were washed 4 times, 100 μL of TMB substrate solution were added to each well, and the samples were allowed to react with the labeled antibody at room temperature for 15 minutes in the dark. The reaction was stopped by adding stop solution, and the absorbance was measured at 450 nm with a microplate reader (Bio-Rad).

Serum calcium, interleukin 17α, tumor necrosis factor α, and interleukin 6 enzyme-linked immunosorbent assays

All serum calcium levels were measured with the Chemistry Analyzer IDEXX VetTest (IDEXX Laboratories). Interleukin 17α (IL-17α), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) levels were quantified using commercial ELISA kits according to the manufacturer's instructions (BioLegend).

Immunohistochemical testing

Collected tissues of omentectomy and partial pancreatectomy, which contained the microcapsules (for both the microencapsuled Ptc and the empty microcapsule transplant groups) and freely floating microcapsules from rat peritoneal cavity, were collected at week 12 and at weeks 50 to 52 (end of the experiment) after the transplant procedure for each group. Frozen tissue sections were then stained with hematoxylin and eosin. In addition, 2 sections of each animal were scored with the use of an image analysis system (Nikon Instruments). Sections were graded by using a grading scale: grade 0 indicated no fibrosis (no fibroblast and/or collagen fibers), grade 1 indicated low-level fibrosis (low number of fibroblast and/or collagen fibers), grade 2 indicated moderate-level fibrosis (moderate fibroblast and/or collagen fibers), and grade 3 indicated severe fibrosis (high number of fibroblast and/or collagen fibers). Each grading represented the average value of 3 capsules per section.

Scanning electron microscopy

Before transplant, microencapsulated Ptcs were visualized by field emission gun scanning electron microscopy using a QUANTA 450 field emission gun (FEI). Scanning electron microscopy was performed at the Science and Technology Center at Bulent Ecevit University.

Statistical analyses

All data collected during the study period (64 weeks in total) were compared, with statistical comparisons made with unpaired Mann-Whitney *t* tests. For time-interval groups, we used a 2-way analysis of variance or comparable mixed-effects analysis followed by a Tukey post hoc test (when there was a missing value) or Sidak's or Dunnett's multiple-comparisons test. In addition, ELISA data were calculated by using the sigmoidal 4-parameter logistic model. All data were visualized with the use of GraphPad Prism software version 8.3. $P < .05$ was considered statistically significant.

Results

All recipient animals (n = 32) were euthanized, with data derived from the 4 groups: microencapsulated Ptc transplant group, Ptc transplant group, empty microcapsule transplant group, and sham group.

Sham group outcomes

The sham group only received injection of isotonic saline solution after laparotomy and were followed for 44 weeks. The sham group exhibited alterations

in food intake and weight loss, which led to early use of euthanasia before the other groups.

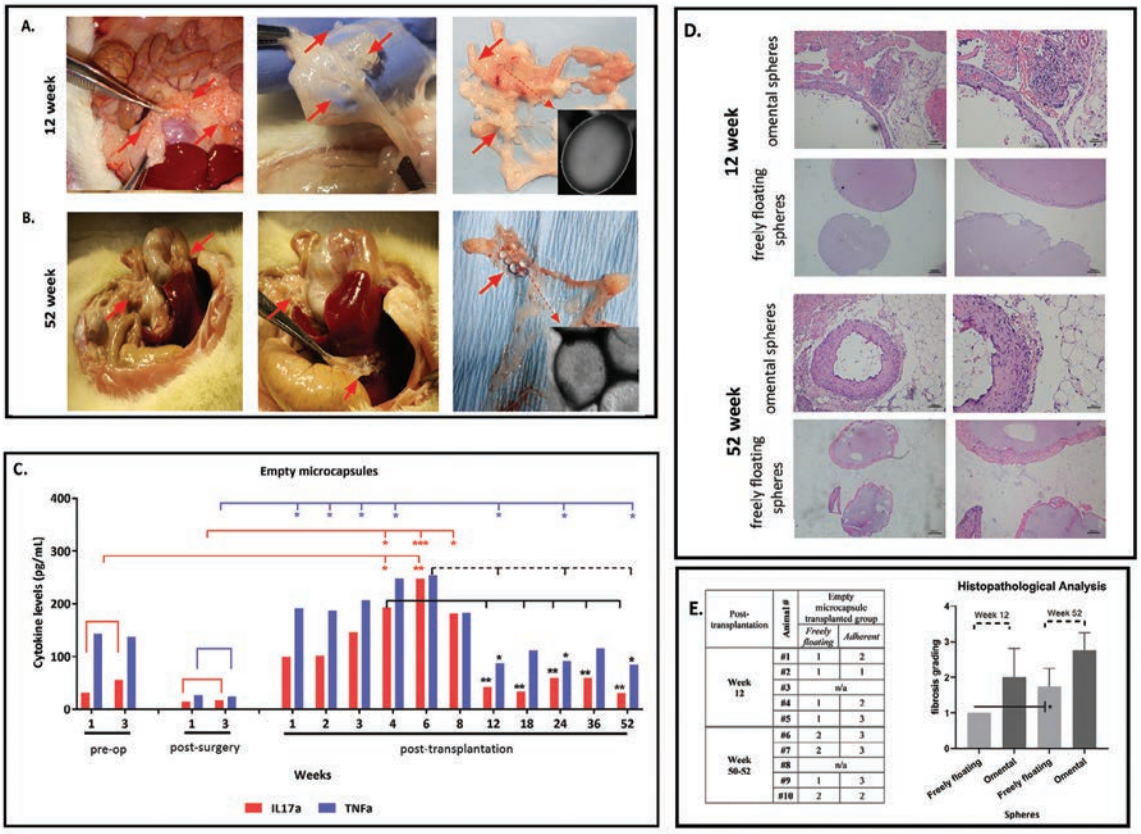
Empty microcapsule transplant group outcomes

At week 1 posttransplant, 1 animal in the empty microcapsule transplant group did not recover after surgery. After transplant, 4 animals in this group were killed and omentectomy and partial pancreatectomy were performed for macroscopic and immunohistochemistry evaluations at week 12 posttransplant (Figure 2A). One animal died at week 28 posttransplant; immediate autopsy showed peritoneal adhesion. Four empty microcapsules had adhered to the liver, and immunohistochemistry was performed (Figure 3). Liver sections showed that neutrophil and leukocyte-rich inflammation was present in the portal area and around the hepatic veins. At week 52, the remaining animals (n = 4) were killed (Figure 2B). Furthermore, omental spheres were enveloped by fibrotic cells with an increasing rate. Despite these multicellular fibrotic layers, freely floating empty microcapsules showed less surrounding cell envelopment even at 1 year after transplant (Figure 2D). Immunohistochemistry evaluation and grading of omental and partial pancreas specimens were performed for both tissues containing omental spheres and the freely floating spheres at week 12 and 52, respectively. When time intervals were compared between groups, fibrosis rate only increased significantly from week 12 to 52 in the freely floating group ($P = .0286$) but not in the group with omental adhered spheres ($P = .3714$) (Figure 2E).

Microencapsulated parathyroid cell transplant group

Before surgery, Ptc were isolated and cultured. Results for cell viability and PTH release level were

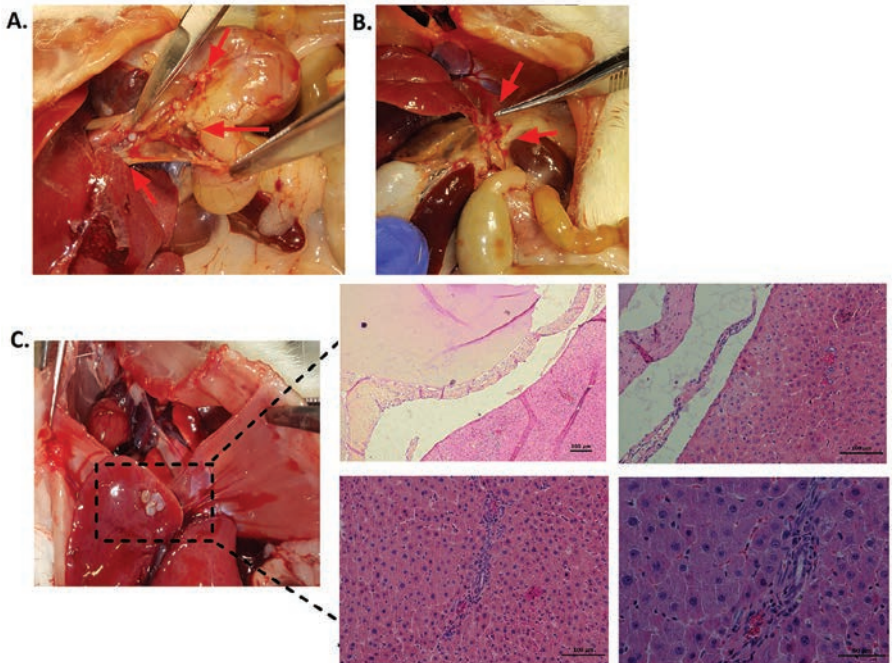
Figure 2. Representative Surgical Images of Empty Microcapsule Transplant Group, Cytokine Levels in Serum Samples, and Stained Sections of Spheres



Abbreviations: IL17a, interleukin 17α; pre-op, preoperative; TNFa, tumor necrosis factor α

(A and B) Comparision of 2% ultrapure alginate adhesion for omental organs between week 12 and 52. Insets show explanted capsule (red dashed arrows). **(C)** Cytokine levels for the empty microcapsule transplant group throughout 52 weeks. IL-17α levels significantly increased at weeks 4 to 8. TNF-α levels increased significantly posttransplant. During the posttransplant period, IL-17α levels dramatically decreased at weeks 12 to 52 compared with that shown at posttransplant week 4 (black straight lines). A similar decline in TNF-α levels at weeks 12, 24, and 52 occurred (black dashed lines). Data are shown as means ± SD. $^{*}P < .05$, $^{**}P < .005$, $^{***}P < .0001$. **(D)** Hematoxylin and eosin-stained sphere histology. **(E)** Histopathological fibrosis grading of the collected microcapsules from the peritoneal cavity and omental sections. Fibrosis significantly increased posttransplant for freely floating microcapsules ($P = .0286$).

Figure 3. Adhered Empty Microcapsule Immunohistochemistry Results



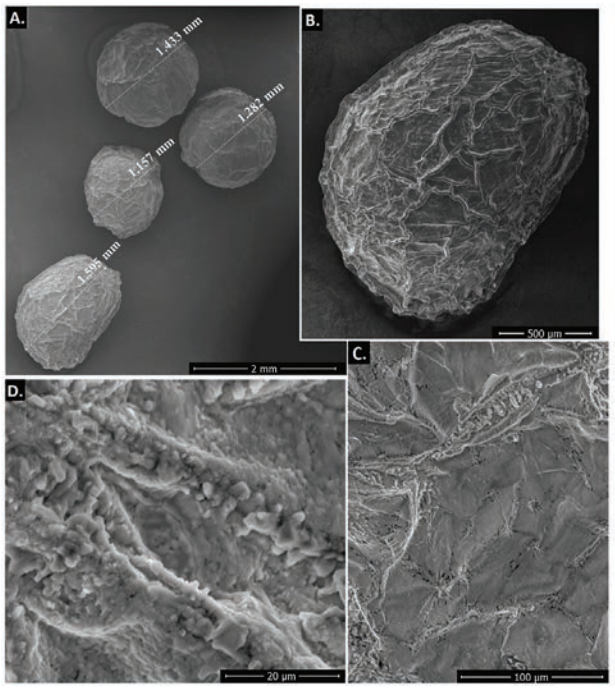
(A and B) One animal underwent macroscopical evaluation. During the autopsy, intraperitoneal adhesion was observed (red arrows). **(C)** Hematoxylin and eosin-stained sections of the liver showed adhered empty microcapsules (black dashed lines) from the empty microcapsule transplant group at week 28 posttransplant. Immunohistochemistry-stained sections of liver sections showed neutrophil and leukocyte-rich inflammation in the portal area and around the hepatic veins.

94.3% and 1270 pg/mL, respectively. Analyses of scanning electron microscopy images showed that the microencapsulated Ptc sphere diameter ranged from 1.28 to 1.59 mm (Figure 4). After transplant, half of the animals were killed and evaluated macroscopically at week 12 posttransplant. We did not detect any unexpected adherence inside the peritoneal cavity, and the morphology of recovered microencapsulated Ptc spheres remained stable. At week 52 (endpoint of the study), the remaining animals were killed. During extraction from the omentum, two-thirds of the transplanted microcapsules were freely floating inside the peritoneal cavity. In the microencapsulated Ptc transplant group, fibrotic cellular overgrowth on the capsule surface of the freely floating and omental spheres was demonstrated, which was surrounded with a varied range of fibrotic cells (data presented for week 52). Retrieved spheres were graded by immunohistochemistry fibrosis grading, with fibrosis rate not found to be significant (Figure 5). As shown in the histological data in Figure 5, the Ptc stained faintly, and freely floating spheres contained less Ptc.

Parathyroid cell transplant group

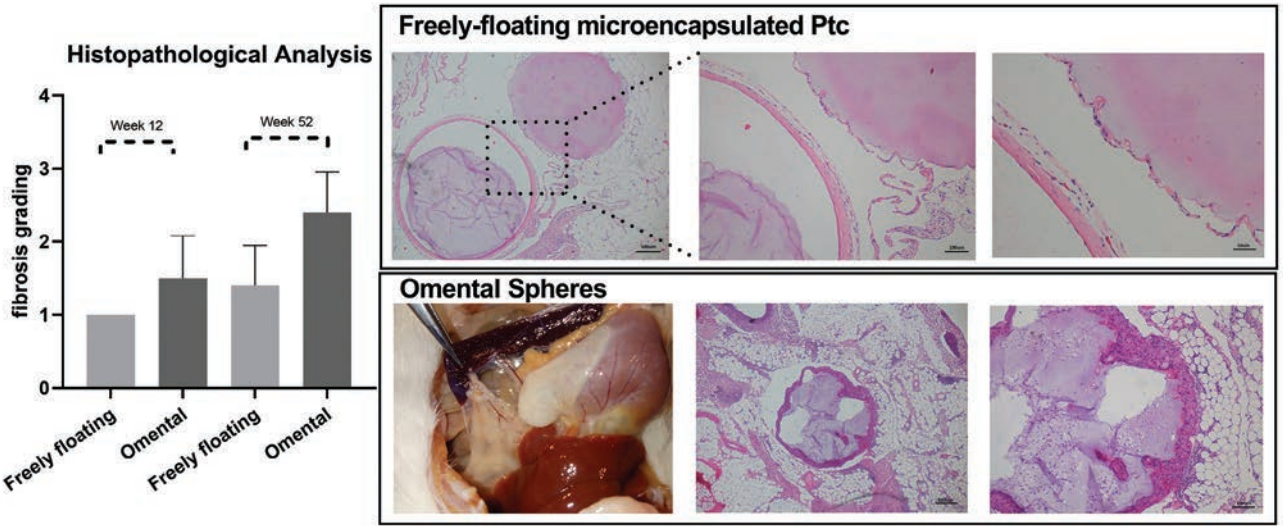
One animal failed to recover at week 1 posttransplant.

Figure 4. Scanning Electron Microscopy Images Illustrating Microencapsulated Parathyroid Cells With Ultrapure Alginate Before Transplantation



(A) Magnification ×50, 5.00 kV, 2 mm of working distance. Lines demonstrate the perimeter of each microencapsule section circumscribed area. (B) Magnification ×130, 5.00 kV, 500 µm of working distance. **(C)** Magnification ×1000, 13 kV, 100 µm of working distance. (D) Magnification ×4000, 13 kV, 20 µm of working distance.

Figure 5. Histopathological Analysis of Spheres and Stained Sections of Microencapsulated Parathyroid Cell Transplant Group After Transplant



Abbreviations: Ptc, parathyroid cells

Graph shows histopathological fibrosis analysis. Changes in fibrosis were not statistically significant ($P > .05$). Data are shown as means ± SD. Freely floating sphere and omental sphere magnifications are shown as ×10 and ×4 and ×4 and ×10, respectively.

At 12 and 52 weeks after xenotransplant, animals were killed and macroscopically evaluated. There were no peritoneal adhesions inside the cavity.

Rat parathormone levels

Rat PTH levels were determined with the use of sandwich ELISA. The results for sham and empty microcapsule transplant group performed as first group, and the microencapsulated Ptc transplant and Ptc-only transplant groups were evaluated as second group. Assay sensitivity and threshold levels were accepted based on nontransplant (sham) group data of each preoperative, postsurgery, and posttransplant time interval separately. Rat PTH levels were 19.84 and 20.65 pg/mL at preoperative week 1 and week 3, respectively. After surgery, levels dramatically decreased for all groups as expected. During posttransplant, these levels remained below 6.29 pg/mL, which is lower than the average threshold level (7.4 pg/mL) (Figure 6A).

Human parathormone levels

Human PTH levels were evaluated for the sham and empty microcapsule transplant group, for the Ptc-only transplant group, and for the microencapsulated Ptc transplant group. Assay sensitivity and threshold levels were accepted based on the empty microcapsule transplant group data of each preoperative, postsurgery, and posttransplant time interval separately.

The preoperative hu-PTH levels were below 2.1 pg/mL for each group; during postsurgery, the hu-PTH levels remained below 3.46 pg/mL. During the posttransplant period, the microencapsulated Ptc transplant group showed functionality after week 3. Subsequently, the hu-PTH level started to decrease at week 18, with the same reduction continuing through to the endpoint of the experiment (week 52); thereafter, hu-PTH levels remained above the accepted threshold level.

Only the Ptc-only transplant group showed a significant increase starting from week 1 post-transplant; this level remained higher than in the other groups even at week 52 (Figure 6B).

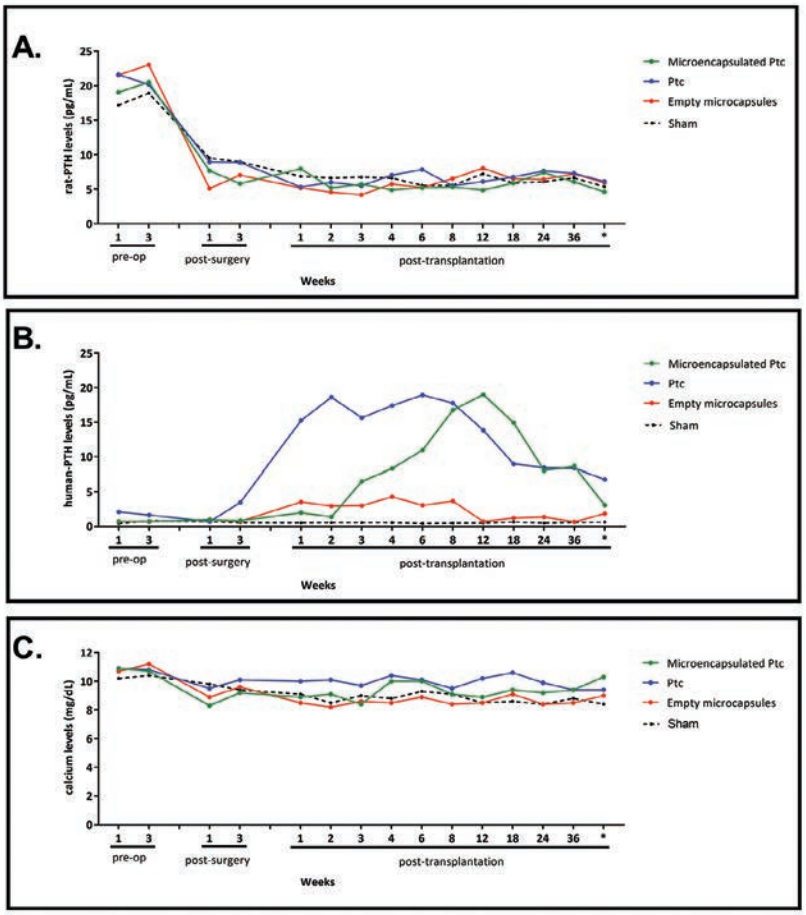
Calcium levels

Before surgery, all animals were normocalcemic, with average calcium level of 9.1 to 10.7 mg/dL; levels were elevated with an average of 0.5 to 0.7 mg/dL throughout all experimental processes (Figure 6C). With regard to physiological function of calcium levels, rats in the Ptc transplant and microencapsulated Ptc transplant groups lost weight; this was not found to be significant because results were not within the exclusion criteria and there were no changes in the food intake.

Cytokine levels

For cytokine evaluation, IL-17α and TNF-α levels were measured in the empty microcapsule transplant group throughout 56 weeks. We found IL-17α levels to be significantly increased at weeks 4 to 6 and at

Figure 6. Serum Parathormone and Calcium Levels Over Time



Abbreviations: pre-op, preoperative; Ptc, parathyroid cells; PTH, parathormone
(A) Rat PTH levels **(B)** Human PTH levels. **(C)** Calcium levels. *Indicates 44-week follow-up period for sham group and 50- to 52-week follow-up period for microencapsulated Ptc, Ptc, and empty microcapsule groups.

weeks 6 to 8 compared with the preoperative and postsurgery levels, respectively. After transplant, IL-17α levels were dramatically decreased at week 12 through 52 compared with posttransplant week 4. Moreover, TNF-α levels were increased significantly posttransplant compared with postsurgery levels. A similar decline at 12, 24, and 52 weeks occurred compared with posttransplant week 6. Cytokine levels are shown in Figure 2C.

For the Ptc-only and microencapsulated Ptc transplant groups, IL-17α, TNF-α, and IL-6 levels were measured in each animal throughout the 64-week study period and compared with levels in the nontransplant group (sham).

In the sham group posttransplant, IL-6 levels were significantly increased compared with our examination of TNF-α levels, particularly at week 18 (P = .0054). After this time point, the animals had reduced food and water intake and displayed some pain-like movements. At week 36, the animals started to lose weight; at week 44, the animals were euthanized (Figure 7A).

In the microencapsulated Ptc transplant group, during the posttransplant period fromweek 1 to week 6, IL-17α levels were relatively high. At week 6 posttransplant, TNF-α levels reached a maximum level, showing a significant difference (P = .0131). Afterward, TNF-α levels gradually decreased and remained below the average range. In addition, IL-6 levels reached their highest level at week 4, although IL-6 levels were relatively stable throughout the 58-week period (Figure 7B).

In the Ptc-only transplant group, no significant differences were detected for TNF-α, IL-17α, and IL-6 throughout the 58-week period. Interestingly, at posttransplant week 8, IL-17α changes were statistically significant compared with IL-6 changes (P = .033) (Figure 7C).

We also analyzed cytokines separately at week 18 posttransplant, with IL-6 levels being statistically significant between the microencapsulated Ptc and Ptc-only transplant groups ($P = .0016$) (Figure 7D).

Discussion

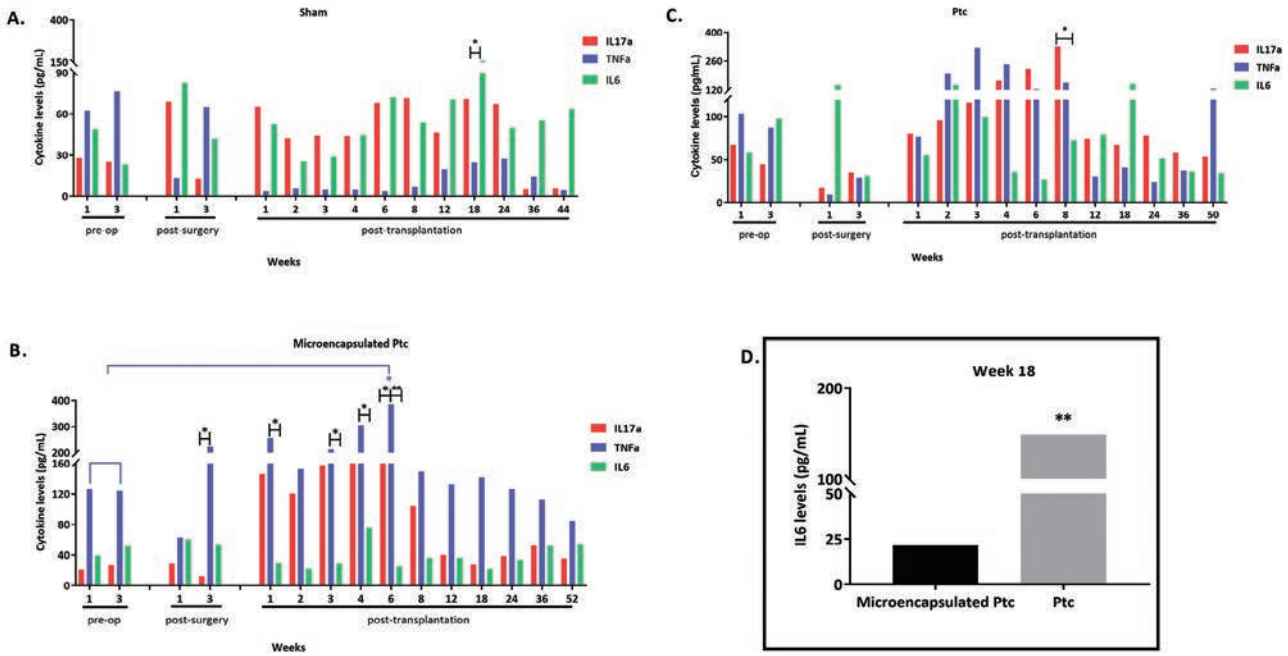
Treatment strategies for autoimmune disorders have so far made slow progress. Ongoing and future studies into investigations of promising treatment options are needed as there are yet no specified therapies.[17]

Hypoparathyroidism is mostly iatrogenic and is rarely congenital (and may also be caused by an autoimmune processes.[1,2,6,18] The management of hypoparathyroidism relies upon symptomatic treatment.[4] Parathyroid allotransplantation may be an alternative therapeutical.[4,5] In a study of the largest number of reported cases, which included 316 allo- and redo-PTX that were performed between 1996 and 2017, varying degrees of success were reported.[5] The necessity of immunoisolation requires more definitive choices, such as allotransplantation by biocompatible materials to avoid immune response.

Graft rejection represents a major drawback in transplantation. To overcome rejection, studies have described different methods and a variety of in vitro and in vivo experiments for PTX.[9,14,19-26] Xenotransplantation triggers an inflammatory pathway, and this leads to a limitation of graft survival.[27] The immunological barriers of allotransplantation and xenotransplantation differ with regard to the transplanted grafts from either cells or organs. However, grafts consisting of cells are exposed more directly to the recipient's immune system.[27,28] Currently, immunoisolation with the use of polymeric materials is one of the contributions to the field of transplantation.[28,29]

Another challenge for PTX is determining the most favorable transplant site. With xenografting of parathyroid tissue/cells, several in vivo routes have been used, including intramuscular,[19,20,30] subcutaneous,[31] and intraperitoneal[32-34] injection. Notably, in a previous study from Nawrot and colleaugues, the peritoneal cavity served as a natural incubator for parathyroid xenotransplantation.[34] Another study reported more effective results when

Figure 7. Cytokine Levels Over Time



Abbreviations: IL, interleukin; pre-op, preoperative; Ptc, parathyroid cells; TNFa, tumor necrosis factor α

(A) Cytokine levels in sham group (n = 2) over 53 weeks in total. IL-6 levels were significantly increased compared with TNF-α at week 18 ($P = .0054$) posttransplant. **(B)** Cytokine levels of microencapsulated Ptc transplant group. TNF-α levels were significantly increased at week 3 postsurgery ($P = .0185$) compared with IL-17α; the same increment occurred at week 6 compared with both IL-17α and the preoperative values ($P = .0189$, $P = .0131$, respectively). TNF-α levels were significantly higher than IL-6 levels during weeks 1, 3, 4, and 6 posttransplant ($P = .0122$ for week 1, $P = .0442$ for week 3, $P = .0101$ for week 4, and $P < .001$ for week 6). **(C)** Cytokine levels of Ptc transplant group. IL-17α levels were significantly higher than IL-6 levels at week 8 ($P = .0330$) posttransplant. **(D)** IL-6 levels were statistically significant at week 18 ($P = .0016$). *$P < .05$; **$P < .005$.

transplanted intraperitoneally.[35] In addition, in vivo studies have reported longer survival rates for islets.[36,37] Therefore, in this study, we prefered use of the omentum to particularly aim for long-term graft function.

Encapsulated PTX has been described in only 7 case reports for 12 recipients.[7,8,21,23,38-40] Hasse and colleagues performed the first microencapsulated PTX for 2 recipients and reported 3-month graft survival.[21] Zimmerman and colleagues performed PTX for 1 recipient. After 3 months, the implantation site of the recipient showed no trace of parathyroid tissue particles or microcapsules.[8] In a later study, in which Khryshchanovich and colleagues transplanted macroencapsulated parathyroid tissue particles and reported 3-month follow-up data, the group demonstrated a nonspecific inflammatory response to the microcapsules.[40] The last case report on microencapsulated Ptc transplantation was performed by Yucesan and colleagues.[23]

To the best of our knowledge, this is the first and the longest follow-up in vivo study on encapsulated parathyroid cells. Several in vivo approaches have revealed that use of Ptc rather than tissue is useful[32] and feasible.[9,19,20,22,33,41] Encapsulation of the parathyroid gland by using semipermeable and biocompatible materials may promote longer survival rates. Hasse and colleagues evaluated different alginate materials and reviewed the results in less than 30 weeks.[9,19-22,41] Other studies have used different encapsulation materials, such as sodium alginate,[26] amitogenic barium alginate,[41,42] barium-alginate-poly-acrylicacid,[32] alginate-poly-lysine-alginate,[33] alginate-poly-lysine-alginate-co-MPEG, and polyvinylidine difluoride.[40] In addition, 1 study demonstrated that mannuronic acid residues stimulated cytokines 10 times higher than guluronic acid.[43] In contrast, another study reported that calcium cross-linked alginate promoted IL-1β secretion in vivo within the first 12 hours after subcutaneous injection.[44] Nontheless, there has been limited mention of the other features of alginate, such as the cross-linking degree, rigidity, or permeability of the alginate capsules in vivo.[12] Therefore, we prepared microencapsules with the ultrapure version for higher biocompatibility and to provide egg-box structure for successful pore integrity and vascularization process into the omentum. It is important to emphasize that the lack of studies is a true obstacle for progress in this field. With this insight, we should be able to demonstrate the methodological details to overcome the current lab-to-lab variations.

Two main goals were planned for this study. The first goal was to investigate the empty ultrapure alginate sphere stability and the adherence capability into the omental tissue and to follow the inflammatory status. We first optimized the in vitro Ptc isolation process and continued to determine the percentage of ultrapure alginate and the cell mass per 27 to 30 microspheres in vitro.[14] Later, we sought to investigate the adherence capability of omental tissue. During the sphere retrieval process, >65% of the spheres had adhered to the omentum, and the remaining capsules were freely floating inside the peritoneal cavity. The fibrosis grade was found to be significantly higher in the freely floating microspheres. The inflammatory response (TNF-α and IL-17α) also increased significantly for up to 6 weeks. We also compared xenotransplant graft longevity by determining PTH and preinflammatory cytokine (TNF-α, IL-17α, and IL-6) levels. The pulsatile secretion of PTH has shown intraindividual variability.[45] The basal PTH level from different patients with secondary hyperparathyroidism has its own secretory dynamics.[42] Therefore, we used 1 parathyroid hyperplasia donor for all processes and this was the main limitation in our study of 10 animals per group.

Transplant-associated stress targets inflammatory responses,[46] and previous studies have demonstrated that synergism of certain cytokines, such as TNF-α and IL-6, may inhibit allograft acceptance[47] or IL-6 promotes IL-17α-dependent rejection.[48] Therefore, cytokines were examined to anticipate the possible role of cytokine-mediated elimination/rejection. Both TNF-α and IL-17α levels for all groups were mostly positively correlated. The presence of IL-6 levels were significantly higher at week 18 posttransplant and at same time hu-PTH levels started to decrease but remained above the preoperative levels. We assumed that the inflammatory response did not hinder transport of nutrients or oxygenation even with the development of subsequent per capsular fibrosis. Of note, fibrosis was significantly altered if the spheres did not adhere to the omentum. The nature of the omental tissue could promote this outcome, and this remains to be elucidated.

Our findings concur with the main idea that encapsulation of xenografts provides immunoisolation. The observed delay in hu-PTH release of

microencapsulated Ptc was possibly caused by the physical barrier formed by microencapsules. Prolonged cytokine release possibly accumulated during the fibrotic cellular growth response to microencapsules. We considered that a main feature of ultrapure alginate is allowing free access to exit for only relatively small proteins, in this case hu-PTH (approximately 12 kDa). Based on the decrease in PTH levels, graft rejection occured after almost 1 year. It is possible that secreted cytokines from monocytes and/or T cells may result in the induction of breaking points, as demonstrated at week 18 for IL-6 and PTH levels.

Conclusions

Our study on the potential clinical use of microencapsulated Ptc transplantation to the omental tissue demonstrated that it is suitable for long-term graft survival. This method may similarly be used for a broad range of applications, such as cell-based protein therapies or other cellular transplant procedures. Importantly, clinical studies are required for the therapeutic benefits for hypoparathyroidism in the future.

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