Treatment of Osteoporosis with TheraCyte-Encapsulated Parathyroid Cells: A Study in a Rabbit Model

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Abstract: Background/Purpose: PTH has been used in the treatment of osteoporosis. Implantation of TheraCyte-encapsulated parathyroid cells increasing the BMD of the spine and femur in a rat model was reported in our previous study. The aim of this study was to compare the effect of TheraCyte encapsulating $4x10^7$ or $4x10^6$ live human parathyroid cell on bone mineral density of ovariectomized rabbits. **Methods:** Twenty-seven New Zealand rabbits divided randomly into three groups: (1) TheraCyte encapsulating $4x10^7$ live parathyroid cells as the group A; (2) TheraCyte encapsulating $4x10^6$ live parathyroid cells as the group B; (3) a sham operation as the control group. Rabbits were ovariectomized 1 month prior to the implantation of the TheraCyte. Blood was drawn from the rabbit at the time of implantation and monthly for four months. The BMD of the lumbar spine (L1-L5) and the left femoral bone were measured with dual-energy Xray absorptiometry (DEXA) 1 month after ovariectomy and 3 months after implantation. **Results:** In the control group, both the BMD of the lumbar spine (L1-L5) and the BMD of the femoral bone had decreased significantly 3 months after implantation. In the group A, the BMD of both the lumbar spine and left femoral bone had increased significantly. In the group B, the BMD of both the lumbar spine and the left femoral bone had also increased significantly. Serum iPTH levels were higher in the group A than in the control group. **Conclusion:** Implantation of TheraCyte A-encapsulated $4x10^7$ live parathyroid cells and TheraCyte B-encapsulated $4x10^6$ cells can increase the BMD of ovariectomyized rabbits after 3 months of implantation. Higher serum iPTH were noted in the group A. Implantation of TheraCyte-B encapsulated $4x10^6$ live parathyroid cells could increase the BMD of ovariectomized rabbits and was safe for rabbits weighing 3.0kg in this study.

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1. Introduction

The anabolic effects of PTH (1-34) on the skeleton via the PTH-1 receptor on osteoblasts and bone marrow stromal cells have been well documented in the literature.[1,2,3] PTH has been used in the treatment of postmenopausal osteoporosis, senile osteoporosis in men, and glucourticoid-induced osteoporosis.[1,2,3,4] Although it is effective in the treatment of osteoporosis, daily injection of PTH for a period of 1-2 years is necessary.[5,6,7] An alternative to the daily injection is the transplantation of parathyroid tissues. There are currently several methods available, however, the main obstacle to human parathyroid transplantation is immunological rejection.[8,9] Implantation of TheraCyte-encapsulated live parathyroid cells increasing the BMD of the spine and femur in a rat model was reported in our previous study.[10] The aim of this study was to evaluate the parathyroid function monthly after implantation of TheraCyte-encapsulated parathyroid cells into ovariectomized rabbits and to determine the effect on BMD 4 months after ovariectomy (3 months after implantation).

2. Materials and Methods

Preparation of the Animals

Twenty-seven New Zealand rabbits, 3kg in weight and 3 months of age, were selected for the study. All rabbits were subjected to a bilateral ovariectomy. One month later, rabbits randomly assigned to three groups were subjected to surgical interventions: group 1 rabbits (No=9) underwent a sham operation on their backs as the control group; group 2 rabbits (TheraCyte A; N=9) received an implantation of the TheraCyte encapsulated $4x10^7$ live parathyroid cells into the subcutaneous layer of their backs; group 3 rabbits (TheraCyte B; N=9) underwent an implantation of the TheraCyte encapsulated $4x10^6$ live parathyroid cells. The body weight of each rabbit was measured at baseline and 4 months after the ovariectomy (3 months after implantation of the TheraCyte).

Preparation of PTH Cells

Parathyroid tissues were obtained from patients undergoing surgery for symptomatic secondary hyperparathyroidism. All specimens were cut into pieces less than 7 mm in size and collected in Roswell Park Memorial Institute (RPMI) solution (85%), dimethyl sulfoxide (DMSO) (10%) and fetal calf serum (5%). After step-freezing to -79°C, specimens were stored in liquid ritrogen (-197°C). The animal Use and Care Committee had approved the procedure and

written informed consent was obtained from all patients.

The parathyroid tissue was thawed in a 37°C water bath and minced into small fragments in a medium (DMEM-F-12) (Sigma Chemical Co., St., Louis, MO, USA) and digested for 2 hours at 37°C in media containing collagenase II (1.2mg/ml, Sigma Chemical Co., St. Louis, MO, USA). After centrifugation at 500g and mechanical dispersion, the pellet was resuspended in the complete growth medium (DMEM-F-12 supplemented with 5% calf serum, 1% Nutridoma-SP (Boehing Manheim, Germany), 100u penicillin/ml, 100ug of streptomycin/ml, 1mM Caclz and 0.5 mM Mgclz) and the suspension filtered through 60- and 150-mesh screens. The viability of the detached cells (trypsin/0.06% EDTA) was tested by the trypan blue method. A mixture of 1 ul of cells (density of $4x10^5$/ml) and 5µl trypan blue was prepared and the mixture then placed on a counting chamber to determine the viability ratio (live cells / live + dead cells). The TheraCyte implantable system (Irvine, Calif) was used, or cell encapsulation. Parathyroid cells were passed through a 150-mesh screen, collected and distributed, and then were suspended in RPMI solution at a density of $4x10^7$ live cells /ml in the TheraCyte A group, and at a density of $4x10^6$ live cells/ml in the TheraCyte B group. Using the centrifugation loading method, according to the users manual (TheraCyte), the TheraCyte encapsulating live parathyroid cells were implanted into the subcutaneous layer of the backs of ovariectomized rabbits. TheraCyte devices were comprised of an inner membrane of 30µm thickness of polytetrafluroethylene (PTFE) and with a 0.4µm pore size that prevented the entry of cells into the device and allowed the entry of antibiotics and compliment factors. A laminated outer membrane with a 5µm pore size and 15µm thickness of PTFE improved biocompatibility and induced vascularization. Finally, $4x10^7$ live parathyroid cells in a volume of 1ml were loaded into the 20-µl device (TheraCyte A) and $4x10^6$ cells in a volume of 1ml were loaded into the 20-µl device (TheraCytes B).

Serum calcium, phosphorus, and human intact parathyroid hormone (iPTH) levels

The body weight of each rabbit was measured at baseline and 4 months after the ovariectomy (3 months after implanttion of the Theracyte). At 0-month, 1-month, 2-months and 3-months after implantation, blood was drawn at 3pm from the rabbits' vein to check serum levels of calcium, phosphorus, and human iPTH levels. Thus, they were not fasting parameters. Three months after implantation, half of the rabbits were sacrificed for histological examination of the TheraCyte system and tissues around the TheraCyte, to find parathyroid cells in the TheraCyte, and reaction and fibrosis around the TheraCyte. Half of the rabbits were kept alive until 7 months after ovariectomy to determine the possibility of bone tumor formation, using X-ray examination. Blood was collected in a tube with sodium heparin, and serum was isolated from the blood after centrifugation at 500 rpm for 10 min at 4°C. Serum calcium levels were established by colorimetric determination using the cresolphalein complex, serum phosphorus levels by UV methods, and iPTH levels by means of a radioimmunoassay method using a commercial kit (Nichols Institute Diagnostics, Capistrano, Calif.). Histological examination included hematoxylin and eosin stains and immunohistochemical statins with monoclonal rabbit antibody against human parathyroid hormone (clone 3B3, Dako Denmark; dilution 100). Immunohistochemical staining was performed, using the one-step horseradish-peroxidase technique (Polymer plus, Zymed, USA), after antigen retrieval by microwaving for 7 minutes x 2 times at 95°C to 99°C in citric buffer (1x concentration, PH 6.0; Euroclone, UK) with diaminobenzidine as chromogen on an automatic immunostainer (Opimax plus 2.0, BioGenex, USA).

Dual-Energy X-ray Absorptiometry (DEXA)

The lumbar spine (L1-L5) and left femoral bone were examined by DEXA. BMD (in grams per centimeter square) was determined using a modal Delphi A apparatus (Hologic, Bedford, Mass) at 1 month and 4 months after ovariectomy (3 months after implantation of the TheraCyte).

Statistical Evaluation

Data were expressed as mean ± standard deviation. Statistical analysis was performed using Wilcoxon signed ranks test for two related non-parametric data or paired t-test for two related parametric data, and repeated measures analysis of variance for iPTH, calcium, and phosphorus levels. To determine which group was different from the others, the simultaneous multiple comparison techniques of Bonferroni were used. SPSS (version 10.0, Chicago, IL) software was utilized. A p value less than 0.05 was considered significant.

3. Results

The viability ratio after thawing the cryopreserved parathyroid cells was 55-79%, while that of the fresh tissue was approximately 99-100%. The mean body weight of rabbits before ovariectomy was 3.03±0.22 kg in the control group, 2.82±0.55 kg in the TheraCyte A group and 2.87±0.56 kg in the TheraCyte B group. Four months later, the mean weight of the rabbits was 3.94 ± 0.41 kg in the control group, 4.01±0.25 kg in the TheraCyte A group, and 4.11±0.35 kg in the TheraCyte B group. The increase in body weight 4 months after the ovariectomy was significant in each group (all $p<0.05$). However, there were no

significant differences among groups with regard to body weight at the 4-month examination. The BMD of L1-L5 1 month after ovariectomy was 0.332±0.016 g/cm^2 in the control group, 0.303 ±0.057 g/cm^2 in the TheraCyte A group, and 0.286±0.033 g/cm^2 in the TheraCyte B. Three months after implantation, the BMD of L1-L5 was 0.317±0.020 g/cm^2 in the control group, 0.370±0.028 g/cm^2 in the TheraCyte A group and 0.399±0.054 g/cm^2 in the TheraCyte B group. These increases in BMD were significant in the TheraCyte A group (p=0.011) and TheraCyte B group (p=0.008), and the decrease in BMD was significant in the control group (p=0.011) (Fig. 1a). The BMD of the left femoral bone 1 month after ovariectomy was 0.562 ±0.071 g/cm^2 in the control group, 0.415±0.047 g/cm^2 in the TheraCyte A group and 0.445±0.069 g/cm^2 in the TheraCyte B group. Three months after implantation, the BMD of the left femoral bone was 0.482±0.055 g/cm^2 in the control group, 0.603±0.048 g/cm^2 in the TheraCyte A group, and 0.587±0.054 g/cm2 in the TheraCyte B group. These increases in the BMD of the left femoral bone were significant in the TheraCyte A Group (p=0.008) and the TheraCyte B group (p=0.008), and the decrease in BMD was significant in the control group (p=0.017) (Fig. 1b). Serum iPTH levels was significantly higher in the TheraCyte A group than in the control group (p=0.021). Serum iPTH levels were not significantly higher in the TheraCyte B group than in the control group (p=0.094) (Table 1).

Serum calcium levels at 1 month in the TheraCyte A group were significantly higher than those at ovariectomy in the same group ($p<0.05$) (Table 2), and also significantly higher in the TheraCyte A group than in the control group (p=0.031) (Table 2). Serum phosphorus levels were not significantly different among the three groups (Table 3). X-ray examination did not reveal the presence of bone tumors at 7 months after ovariectomy (6 months after implantation) in twelve rabbits (four in the control group, four in the TheraCyte A group and four in the TheraCyte B group). One month after implantation, the histological examination using hematoxylin and eosin (Fig. 2a) and the immunohistochemical stains (Fig. 2b) revealed the presence of parathyroid cells in the TheraCyte. Three months after implantation, parathyroid cells could be found both in the TheraCyte A group and the TheraCyte B group (Figs. 3a, 3b and 4a, 4b). Few fibroblast cells and no fibrosis were found around the TheraCyte (Figs. 2a, 3a, 4a).

4. **Discussion**

The PTH effect, improving bone mass and strength, has been noted in rats, monkeys, dogs, and rabbits.[11,12,13,14] The largest and most important clinical study to date is that of Neer and Colleagues.[5] At the end of their experimental study period, bone density values in the spine had increased by 9.7% in the 20-µg teriparatide (PTH 1-34) group and by 13% in the 40-µg dose group, compared with a 1% increase in the placebo group. The changes in hip femoral neck BMD were smaller, being 2.8% in the 20-µg group compared with a loss of 0.7% in those participants receiving a placebo. The proportion of women who experienced more than one vertebral fracture was reduced by 90% with the 20-µg daily dose, from 5% to 0.5%.[5] These results demonstrated that teriparatide is very effective in halting the progression of vertebral fractures in women known to have severe osteoporosis. The 40-µg dose was associated with more frequent side effects in the report. The 20-µg dose of triparatide, administered by daily subcutaneous injection, was approved for clinical use in November 2002. PTH (1-34) treatments in rats are associated with an increased incidence of bone tumor.[6] Also, daily injections of PTH (1-34) or PTH (1-84) are inconvenient and troublesome, which is why we initiated our studies. The results of our previous study showed that implantation of TheraCyte-encapsulated live parathyroid cells could increase the BMD of the spine and femur in a rat model.[10] Now, we extended the study to a rabbit model. In our rabbits, body weight at 4 months after ovariectomy was significantly higher than that at the baseline. However, the differences among the groups were not significant at 4 months after ovariectomy. The effect of body weight on BMD could therefore be ignored.[15,16,17] No bone tumors were found in either the control group or the experimental group during a 7-month period. We used parathyroid cells that had been cryopreserved for more than 3 years, yet the viability after thawing was quite good, at around 70%. An approximate 70-90% reduction in live cells has been observed following cryopreservation of bovine parathyroid glands, either as tissue fragments or as dispersed cells, regardless of the length of cryopreservation.[18] However, a higher percentage of viable cells – in excess of 88% -- has been reported in other studies[10,19,20,21] as well as this study. Using a microencapsulation of human parathyroid cells, various investigators have found a significant increase in PTH secretion in vitro and in animals without pharmacological immunosuppression.[22,23,24]

Following the implantation of the TheraCyte-encapsulated parathyroid cells in rabbits, we found that the parathyroid cells could survive and secrete iPTH (1-84) for up to 3 months without immunotherapy. No lymphocyte infiltration was found within the TheraCyte device. Some fibroblast cells were found around the TheraCyte, but without foreign body response. Serum levels of iPTH (1-84) were higher in the TheraCyte A group, which caused

a higher serum calcium level than in the control group due to the greater number of cells in the TheraCyte A group. Serum levels of phosphorus were not significantly different between the groups, because the normal kidney in the rabbit was able to keep the phosphorus levels at normal ranges. Three months after implantation, the BMD of L1-L5 in the TheraCyte A and TheraCyte B group increased significantly. Also, 3 months after implantation, the BMD of the left femoral bone in the TheraCyte A and TheraCyte B group, respectively, increased significantly. By comparison, in the control group, the BMD of L1-L5 and of the left femoral bone decreased significantly due to the ovariectomy effect. Although the results are encouraging, care must be taken when extrapolating these results to human. As with any animal study,[25,26,27,28] not all results seen in lower species are reproducible in higher species. Further sutides[29] are needed to determine the clinical significance. We concluded that implanted TheraCyte-encapsulated $4x10^6$ live parathyroid cells can function very well and increase BMD in both the lumbar spine and femoraup to 3 months or longer. In addition, the TheraCyte-encapsulated $4x10^6$ live parathyroid cells did not cause high levels of calcium or low levels of serum phosphorus. Implantation of TheraCyte-encapsulated $4x10^7$ live parathyroid cells can increase BMD in both the lumber spine and the femoral bone, but it may cause higher serum iPTH and calcium levels due to the greater number of cells in the TheraCyte. Implantation of TheraCyte-encapsulated $4x10^6$ live parathyroid cells increased the BMD of ovariectomized rabbits and was safe for rabbits weighting 3.0kg in this study.

Table 1. Serum levels of iPTH (pg/ml) at 0, 1, 2, and 3 months after TheraCyte implantation

	Ovariectomy	One month	Two months	Three months
Control (n=9)[ab]	10.248±0.88	10.604 ±0.62	9.913± 0.43	10.068 ±1.32
Theracyte A[ac] (n=9)	9.092±1.55	21.325± 12.12*	18.667± 7.43*	17.34± 8.47*
TheraCyte B[bc] (n=9)	8.843±1.37	12.215 ±3.84	14.879± 6.48	15.774 ±8.72

All data = means ± standard deviation.

* P <0.05 versus at ovariectomy (using paired t-test)

[abc] Values were obtained using repeated measures analysis of variance.

[a]p=0.021; [b]p=0.094; [c]p=0.462

TheraCyte A: implantation of $4x10^7$ parathyroid live cells

TheraCyte B: implantation of $4x10^6$ parathyroid live cells

Table 2. Serum levels of calcium (mg/dl) at 0, 1, 2, and 3 months after TheraCyte implantation

	Ovariectomy	One month	Two months	Three months
Control (n=9)[ab]	11.9±1.03	12.2±2.09	12.3± 1.39	12.5± 1.47
Theracyte A[ac] (n=9)	12.3±0.94	13.7± 1.13*	14.6± 1.36*	13.7± 1.50*
TheraCyte B[bc] (n=9)	12.9±0.98	12.8±1.17	13±1.69	12.7± 1.47

All data = means ± standard deviation.

* P <0.05 versus at ovariectomy (using paired t-test)

[abc] Values were obtained using repeated measures analysis of variance.

[a]p=0.031; [b]p=0.066; [c]p=0.711

TheraCyte A: implantation of $4x10^7$ parathyroid live cells

TheraCyte B: implantation of $4x10^6$ parathyroid live cells

Table 3 Serum levels of phosphorus (mg/dl) at 0, 1, 2, and 3 months after TheraCyte implantation

	Ovariectomy	One month	Two months	Three months
Control (n=9)[ab]	11.9±1.03	12.2±2.09	12.3± 1.39	12.5± 1.47
Theracyte A[ac] (n=9)	12.3±0.94	13.7± 1.13*	14.6± 1.36*	13.7± 1.50*
TheraCyte B[bc] (n=9)	12.9±0.98	12.8±1.17	13±1.69	12.7± 1.47

All data = means ± standard deviation.

* P =0.156 versus at ovariectomy (using paired t-test)

** P=0.440 versus at ovariectomy (using paired t-test)

[abc] Values were obtained using repeated measures analysis of variance.

[a]p=0.156; [b]p=0.440; [c]p=0.491

TheraCyte A: implantation of $4x10^7$ parathyroid live cells

TheraCyte B: implantation of $4x10^6$ parathyroid live cells

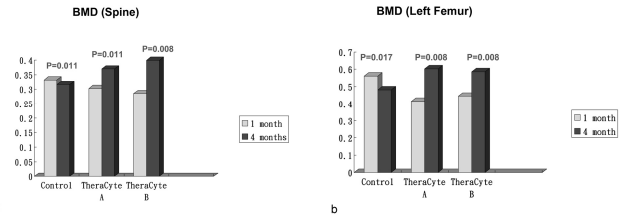

Fig.1 1a-bBone mineral density (BMD) after ovariectomy and 3 months after implantation (4 months after ovariectomy) in the control, and TheraCyte (a) $4x10^7$ live parathyroid cell and (b) $4x10^6$ live parathyroid cell groups. Wilcoxon Signed Ranks test was used.

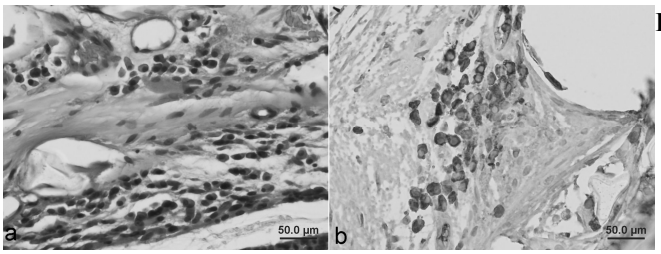

Fig. 2 (a) Hematoxylin and eosin staining reveals the presence of cells in the TheraCyte (x132) group 1 month after implantation of the TheraCyte-encapuslated $4x10^6$ live parathyroid cells. (b) Immunohistochemical staining reveals the presence of iPTH-positive cells in the TheraCyte (x132) group 1 month after implantation of the TheraCyte-encapsulated $4x10^6$ live parathyroid cells.

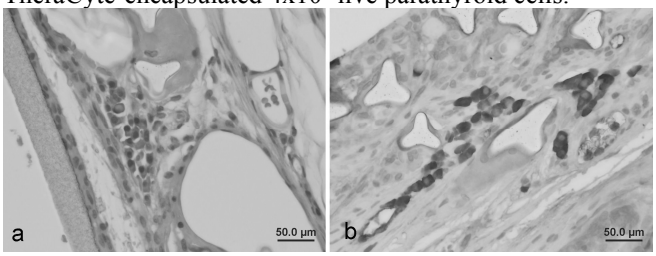

Fig. 3 (a) Hematoxylin and eosin stain reveals the presence of cells in the TheraCyte (x132) group 3 months after implantation of the TheraCyte-encapsulated $4x10^6$ live parathyroid cells. There is no fibrosis and few fibroblast cells around the TheraCyte. (b) Immunohistochemical staining reveals the presence of iPTH-positive cells in the TheraCyte (x330) group 3 months after implantation of the TheraCyte-encapsulated $4x10^6$ live parathyroid cells. There are fewer iPTH cells 3 months after implantation than at 1 month after implantation(Fig. 2b).

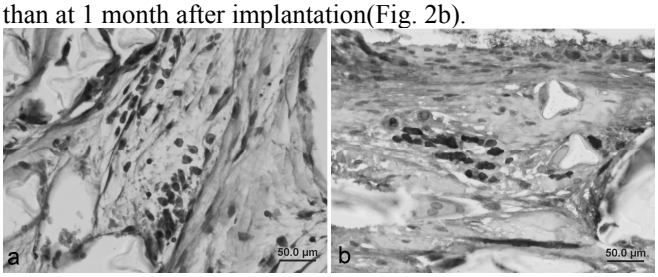

Fig. 4 (a) Hematoxylin and eosin staining reveals the presence of cells in the TheraCyte (x132) group 3 months after implantation of the TheraCyte-encapsulated $4x10^7$ live parathyroid cells. There is no fibrosis and few fibroblast cells around the TheraCyte. (b) Immunohistochemical stain reveals the presence of iPTH-positive cells in the TheraCyte (x132) 3 months after implantation of the TheraCyte-encapsulated $4x10^7$ live parathyroid cells. There are fewer iPTH cells 3 months after implantation than 1 month after implantation (Fib. 2b).

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