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Cellular changes following direct vitamin D injection into the uraemia-induced hyperplastic parathyroid gland

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Abstract

Background. Hyperplasia of the parathyroid gland (PTG) is associated not only with excessive secretion of parathyroid hormone (PTH) but also with changes in the parathyroid cell (PTC) characteristics (i.e. hyperproliferative activity and low contents of vitamin D and calcium-sensing receptors). The control of PTG hyperplasia is most important in the management of secondary hyperparathyroidism (SHPT), because the advanced stage of hyperplasia is considered irreversible. For the better control of the PTH level in dialysis patients with such advanced SHPT, percutaneous vitamin D injection therapy (PDIT) under ultrasonographic guidance was developed and various cellular changes caused by this treatment were also investigated using an animal model.

Methods. The PTGs of Sprague–Dawley rats, which had been 5/6-nephrectomized and fed a high-phosphate diet, were treated with the direct injections of vitamin D agents, and cellular effects focusing the above-mentioned characters were investigated.

Results. An adequacy of the direct injection technique into the rats' PTGs and the successful effects of this treatment in various biochemical parameters were confirmed. Such characteristics of advanced SHPT were simultaneously improved; in particular, it was confirmed that this treatment may be effective in controlling PTG hyperplasia by, at least in part, apoptosis-induced cell death.

Conclusions. A locally high level of vitamin D strongly may suppress PTH secretion and regress hyperplasia, which is involved in the induction of apoptosis in PTCs, based on the simultaneous improvements of cellular characters of advanced SHPT. The PTH control introduced by this treatment successfully ameliorated osteitis fibrosa (high bone turnover rate).

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Introduction

Secondary hyperparathyroidism (SHPT) is a common complication of chronic kidney disease (CKD) and is characterized not only by a high serum level of parathyroid hormone (PTH) but also by hyperplasia of the parathyroid gland (PTG). In CKD, PTH plays a very important role in bone and mineral metabolisms. The abnormalities in bone in the setting of CKD include the effects of high levels of PTH on bone, which results in the high-turnover bone disease osteitis fibrosa. In addition, the effects of both absolute and relative lack of PTH lead to a different skeletal abnormality known as adynamic bone, which is characterized by an extremely low bone turnover. It was reported that the relationship between bone fracture and PTH level showed a U-shaped curve, in other words that both low and high levels of PTH carried high risk of bone fracture [1]. Moreover, the incidence of bone fracture is associated with an increased risk of mortality in the dialysis population and it is well known that SHPT may contribute to vascular calcification and potentially thereby worsen the prognosis in patients with CKD [2–4]. Thus, it is important to control the PTH level, not only for appropriate bone and mineral metabolisms but also for potential improvement of the prognosis in patients with CKD.

Under uraemic conditions, phosphorus (P) retention and the low levels of calcium (Ca) and active vitamin D play very important roles in the progression of PTG hyperplasia. As medical treatments for SHPT, P binders, as well as calcitriol, and its analogue are effective in suppressing both the serum level of PTH and progression of PTG hyperplasia in the early stages, but advanced SHPT with a severely hyperplastic PTG is resistant to the above-mentioned treatments

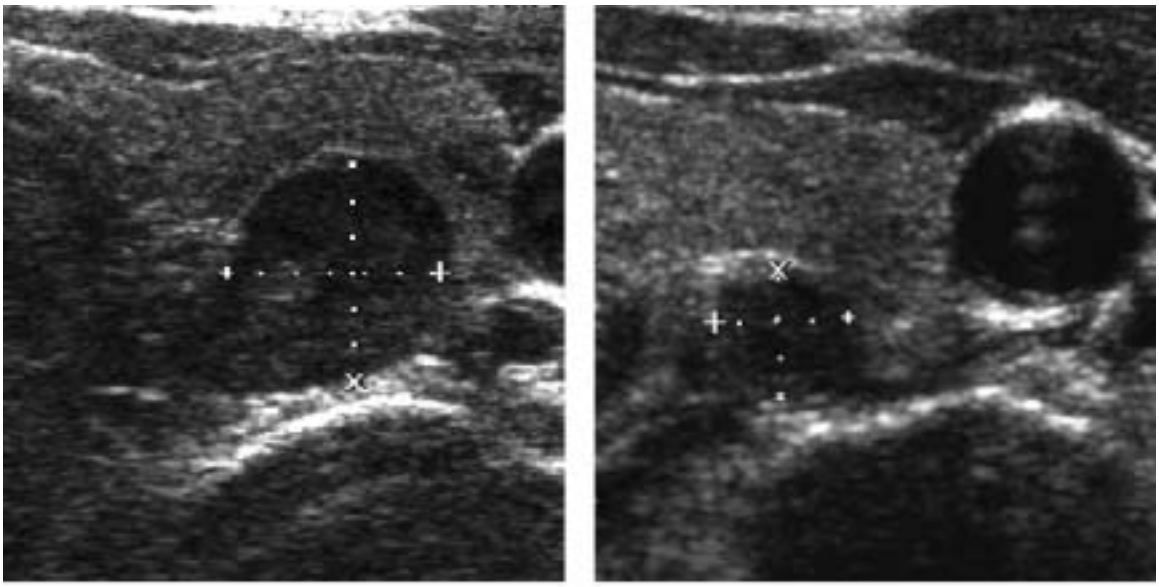

Fig. 1. Changes in the ultrasonographic image of the PTG following PDIT. Before: Before PDIT; After: 12 weeks after PDIT.

because of the low contents of vitamin D and Ca-sensing receptors (VDR and CaSR, respectively) in the parathyroid cells (PTCs) [5,6]. Thus, it is most important to pay attention to these factors, which affect resistance to medical treatments for SHPT, in particular PTG hyperplasia, when controlling SHPT.

When SHPT progresses into such an advanced status, it is considered irreversible. Thus, patients with advanced SHPT require the surgical removal of PTG (PTX) or percutanous ethanol injection therapy (PEIT) for the mass reduction of PTG. For the safe control of SHPT in such patients, a novel therapeutic tool, percutaneous direct vitamin D injection therapy (PDIT) was developed. The patients, who satisfied the guideline of the Japanese Society for Parathyroid Intervention [7], were treated by the series of six consecutive daily episodes of PDIT. Both serum intact-PTH levels and the PTG volumes following PDIT were significantly decreased. Thus, we considered that one of the mechanisms of the favourable clinical effect was regression of the hyperplastic PTG (Figure 1) [8–10]. In some patients non-severe complications were observed, such as a mild pain accompanied by the injection and mild subcutaneous haemorrhage. In particular, some problems complicating with PEIT were never observed, e.g. injury of surrounding tissues by ethanol leaking from the PTG, such as transient recurrent or sympathetic nerve palsy and severe pain. Therefore, it is considered that PDIT is a safe and effective treatment for advanced SHPT (Table 1).

The mechanisms for explaining the preferable clinical effects of this treatment were investigated using molecular and morphological examinations. At present, there are no available cell lines of PTC for investigating the cellular effects of the target agent; thus, the method of primary culture using the removed PTGs was performed and a relationship was shown between PTC death and various agents using this method [11,12]. However, it is considered that this method is not suitable for the investigation of cell death in particular, due to many non-physiological effects. Thus, an animal model and a method of direct injection into its PTGs were developed and these advantages made it possible to investigate the *in vivo* effects of a highly concentrated agent for PTC of uraemia-induced SHPT.

Developments of an animal model of SHPT rat and the direct injection technique into PTGs

For the animal model of SHPT, we adapted male Sprague–Dawley rats with 5/6-nephrectomy, which were fed a normal diet (0.9% P, 1.12% Ca) for 1 week after this procedure and then switched to a high-P and low-Ca diet (1.2% P, 0.4% Ca) for 8 weeks. At the completion of these procedures, the body weight, haemoglobin (Hb) and ionized Ca (Ca^{2+}) levels were lower and serum blood urea nitrogen (BUN), creatinine (Cr), P and intact-PTH levels were higher than those of normal rats. The PTG of this uraemic rat was severely hyperplastic, and the expression levels of VDR and CaSR in PTC were significantly decreased [13]. Moreover, it was confirmed that the intravenous administration of vitamin D even at a very high dose failed to decrease the serum PTH level in these rats [14]. These results indicated that this animal was appropriate for uraemia-induced advanced SHPT model, which were resistant to medical treatments including vitamin D therapy.

Bilateral PTGs of the rats were exposed surgically and maxacalcitol (OCT: 10 μg/ml; DI-OCT) or its vehicle [phosphate buffer containing 0.01% polyoxyethylene sorbitan monolaurate and 0.2% ethanol (pH 8.0, isotonic solution)] (DI-vehicle) was directly injected using a 30-gauge

Table 1. Clinical difference between PEIT and PDIT

	PEIT	PDIT
Benefit	1. Effective in reduction of the PTH level 2. Achievement of the target PTH level by the repeated treatments 3. Low risk of hypoparathyroidism 4. Not required any anaesthesia	
	1. Visibility of the injected solution at real time	1. No complication of recurrent and sympathetic nerve palsy 2. Allowed bilateral treatments
Fault	1. Occasionally not sufficient reduction of the PTH level 2. Limitations caused by not typical location of gland 3. Subcutaneous haemorrhage	
	1. Recurrent or sympathetic nerve palsy 2. Severe local pain during injection 3. Not allowed bilateral treatments 4. Difficulty of PTX caused by the adhesion of the PTG with surrounding tissues after PEIT	1. Poor effect in a severely advanced status 2. Poor visibility of the injected solution

PEIT: percutaneous ethanol injection therapy; PDIT: percutaneous vitamin D injection therapy; PTX: parathyroidectomy; PTG: parathyroid gland.

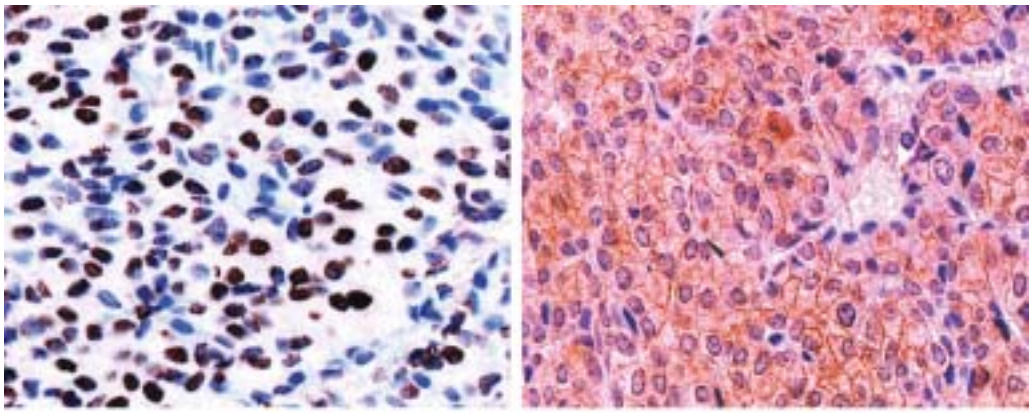

Fig. 2. Immunohistochemical staining of VDR and CaSR in PTCs following DI-OCT.

(G) needle (specially made by Tochigi Seikou Co., Inc., Tochigi, Japan) under a zoom stereo microscope. The needle tip is blind and a one-side hole exists for manoeuvring it to the gland's centre. Immediately after the injection, the solution that had leaked from the PTG was washed away with saline. These procedures were performed under diethyl ether inhalation. The appropriateness of the method was confirmed by the results of direct injection of both Indian ink and [26-^{3}H]-OCT (^{3}H-OCT), and the actual volume of solution injected was similar to the original volume of the PTGs of this rat model (2.47 $\pm$ 0.65 μL/PTG; $N = 10$) [13,15].

Specific effects of DI-OCT in PTC of uraemia-induced SHPT model rat

The development of the animal model of advanced SHPT and the method of direct injection into the PTG have made it possible to investigate the cellular effects of PDIT in detail. Time course changes of VDR and CaSR mRNA expressions determined by reverse transcription-polymerase chain reaction (RT-PCR) and the immunohistochemical expressions in PTGs following DI-OCT were investigated. The expression levels of both VDR and CaSR in PTGs following DI-OCT were significantly higher than those before injection and at the corresponding time after DI-vehicle (Figure 2). Moreover, for confirmation of the functional activity of upregulated CaSR following DI-OCT, the changes in four parameters calculated from the model of Brown [16] in the Ca–PTH response curve were investigated. Not only maximum PTH levels but also the set point after DI-OCT significantly decreased compared with those before DI-OCT. The sigmoid curve clearly shifted to the left and downward following the DI-OCT (Figure 3). However, these findings were not observed after DI-vehicle [13]. These results indicate that this treatment can make it possible to appropriately control both Ca and PTH levels. In an experimental rat advanced SHPT model, kidney transplantation normalized serum PTH, Ca, P and urea levels but did not upregulate VDR and CaSR mRNA expressions in the PTG [17]; however, DI-OCT could functionally improve these sensitivity of PTCs.

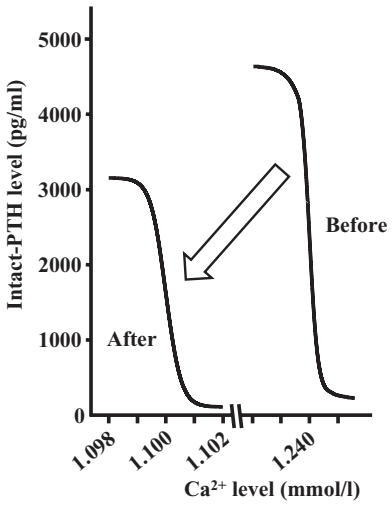

Fig. 3. Changes in the Ca–PTH response curve following DI-OCT. The means of following parameters are fitted to the Brown's equation [16]: intact-PTH $= (a - d)/(1 + (Ca^{2+}/c)^b) + d$, where c is the set point, d and a are minimum and maximum PTH levels achieved by hypercalcaemia and hypocalcaemia, respectively, and b is proportional to the slope of the Ca–PTH relationship at the set point.

The mechanism of PTG regression following PDIT

In the clinical study, the significant decrease in PTG volume determined by ultrasonographic examination was confirmed and one of the important mechanisms explaining the preferable clinical effects of PDIT was considered to be the regression of PTG hyperplasia. This required a decrease in the number of PTCs, so the cell death caused by DI-OCT was investigated. The PTGs of this advanced SHPT model rats were treated with two consecutive DI-OCT or DI-vehicle. The PTGs were excised 24 h after the final injection and evaluated for PTC apoptosis using light and electron microscopy, TUNEL method and DNA electrophoresis. DI-OCT markedly increased the number of TUNEL-positive PTCs and there was ladder formation on DNA electrophoresis (Figure 4), as well as the characteristic morphological features of apoptosis in both the light and electron microscopic studies: nuclear pyknosis and fragmentation with formation of apoptotic bodies, and intact cell membrane and cytoplasmic organs. These findings were never observed in PTGs following DI-vehicle. The induction of apoptosis in PTC following PDIT was also confirmed by analysing the PTG samples obtained by the biopsy technique before and after PDIT in uraemic patients [9,10]. Interestingly, the induction of apoptosis in PTC following direct injection of all the injectable vitamin D metabolites such as calcitriol, paricalcitol, and doxercalciferol as well as OCT, which were developed for the treatment for SHPT and are clinically used in many countries, was confirmed [13,18]. PTC-apoptosis induced by vitamin D has been controversial for the past few decades [11,19–21] because investigators have not been able to show the characteristic features using the available cellular and molecular biological techniques. In particular, it was considered that conventional intravenous administration of vitamin D, even at very high doses, failed to induce PTC-apoptosis in advanced SHPT (severely hyperplastic PTG), principally because of the limited PTC uptake of vitamin D related to the significantly decreased content of VDR. However, the direct injection technique overcomes this limitation and has been shown to induce PTC-apoptosis, as indicated by results from the TUNEL method, DNA electrophoresis and electron microscopic examination [9,10,13,15,18]. Thus, these findings are convincing evidence that the regression of PTG hyperplasia following PDIT is related to, at least in part, a decrease in the number of PTCs because of apoptosis-induced cell death. However, it is considered that more advanced studies are required to investigate the detailed mechanism about the induction of PTC-apoptosis by vitamin D.

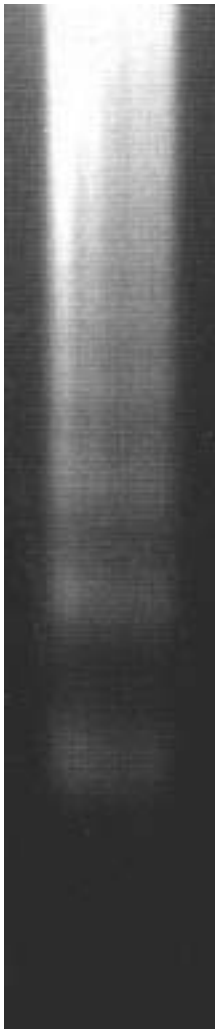

Fig. 4. Detection of DNA fragmentation in the PTG following DI-OCT using 2% agarose gel electrophoresis.

PDIT enables administration of the higher concentrated vitamin D to nuclear vitamin D binding sites of PTC

These preferable cellular effects were not induced by conventional administration of vitamin D. Thus, to explain

the mechanisms of the specific cellular changes in PTCs undergoing DI-OCT, the particular difference in the degree of nuclear localization of OCT between direct injection and systemic administration was investigated using a bioimaging analyser system (BAS) and microautoradiography (mARG) with ^{3}H-OCT. Previous reports showed the methods of these examinations and the evaluation of the results in detail [22,23]. The bilateral PTGs of rat were surgically exposed, and only the left gland was directly injected with ^{3}H-OCT (DI-^{3}H-OCT). The time course of the changes in both radioactivity and localization of ^{3}H-OCT in the bilateral glands was analysed using a BAS and mARG, respectively. A very high dose of unlabelled calcitriol was administered intravenously (IV-1,25D_3) prior to DI-^{3}H-OCT, as a competitive study. Peak radioactivity levels in the directly injected and intact PTG occurred immediately and 1 h, respectively, after DI-^{3}H-OCT, and the difference was about 50-fold higher in the treated gland. The latter level was almost the same as that following intravenous administration of ^{3}H-OCT at a very high dosage, and it was considered that this level indicated the limitation of the OCT uptake into the PTG of this uraemia-induced advanced SHPT model by the conventional administration. The mARG showed a marked concentration of silver grains in the nuclei of PTC in the gland treated with DI-^{3}H-OCT. However, this concentration was significantly suppressed by IV-1,25D_3 prior to DI-^{3}H-OCT. These results indicated that DI-OCT enables the administration of a highly concentrated drug for specific binding to nuclear vitamin D binding sites, including VDR of PTC, which markedly suppresses PTH, improves the response to Ca and vitamin D and induces apoptosis in PTC [15].

Moreover, it was recently reported that very high concentrations of both calcitriol and OCT in the PTG improve abnormal gene expression of PTG, which might directly and/or indirectly be related to PTH synthesis and secretion, and PTC proliferation and sensitivity to medical treatments for SHPT. Such normalizations might contribute to the better control of SHPT following PDIT [24].

Amelioration of skeletal abnormality caused by PTH control based on the specific cellular effects of PDIT

Next, we examined the maintenance of the above-mentioned cellular effects by intravenous OCT administrations following DI-OCT, as well as the histomorphometric alterations in the bone induced in the control of SHPT on the basis of ameliorating these cellular characteristics. The same advanced SHPT model rats were divided into four treatment groups: (1) basic uraemic (at the baseline; basic uraemic group), (2) one injection of DI-OCT followed by intravenous OCT administration for 4 weeks (IV-OCT) (DI-OCT + IV-OCT group), (3) one injection of DI-vehicle followed by the same dose of IV-OCT (DI-vehicle + IV-OCT group) and (4) no treatment for an additional four weeks (uraemic control group). The effects of these treatments on serum intact-PTH level, PTG weight, VDR and CaSR expression levels in PTGs and bone histomorphometric parameters were investigated. In the DI-OCT + IV-OCT group, the significant decrease in the serum intact-PTH level and the upregulations of VDR and CaSR expression levels in PTGs were maintained by the subsequent IV-OCT. A significant decrease in PTG weight compared with that of the basic uraemic group was also observed. In the basic uraemic, DI-vehicle + IV-OCT and uraemic control groups, the findings of severe osteitis fibrosa such as enhanced bone and osteoid formations, hypomineralization of bone, bone erosion with active osteoclasts, double labelling and diffuse and irregular labelling are observed. These findings indicate pathologic bone features associated with advanced SHPT. However, in the bone treated by DI-OCT + IV-OCT, an enhanced bone lamellar structure formation, attenuations of osteoid formation and bone erosion, and regular labelling were noted, and these findings were almost the same as the features of the normal bone (Figure 5) [14].

Previous reports showed that the conventional administration of OCT or another vitamin D analogue mediates the effective prevention of bone disease and ameliorates the established bone histopathology and bone histomorphometric abnormalities in uraemic animals and patients [25–27]. However, the severity of SHPT was mild in subjects of these previous studies, whose serum PTH levels were significantly decreased by the conventional administration of these agents. In general, patients with advanced SHPT, which is unresponsive to conventional medical treatments, undergo the PTX. PTX is very effective in decreasing serum PTH and Ca levels; however, it was reported that this treatment often induces a low bone turnover rate [28,29]. A low turnover and adynamic bone diseases caused by an excessive decrease in the PTH level, particularly after PTX, could affect the progression of arterial calcification [4]. Moreover, PTX can lead to complications, such as the necessity for general anaesthesia, the hyper- or hypofunction of autotransplanted PTGs and psychological distress. DI-OCT can make it possible to appropriately control the serum PTH level based on the normalizations of above-mentioned aetiological factors of advanced SHPT. Moreover, these effects successfully ameliorated osteitis fibrosa (high bone turnover rate). Thus, it is considered that this novel treatment with the appropriate following treatments including the control of P and the conventional administration of vitamin D may contribute to the improvement of the prognosis of dialysis patients with advanced SHPT.

Conclusion

PDIT enables inducement of a significant decrease in PTH levels in patients with advanced SHPT, who require PTX. Moreover, this decrease is maintained by conventional treatments subsequent to PDIT, and one of the mechanisms underlying this favourable clinical effect is simultaneous amelioration of the important aetiological factors that relate to the resistance to medical treatments for SHPT; marked suppression of PTH synthesis and secretion, upregulation of both the VDR and the CaSR and induction of PTC-apoptosis. Thus, it is possible for some patients with very severe SHPT to continue medical treatments and to avoid PTX or PEIT following the introduction of this novel

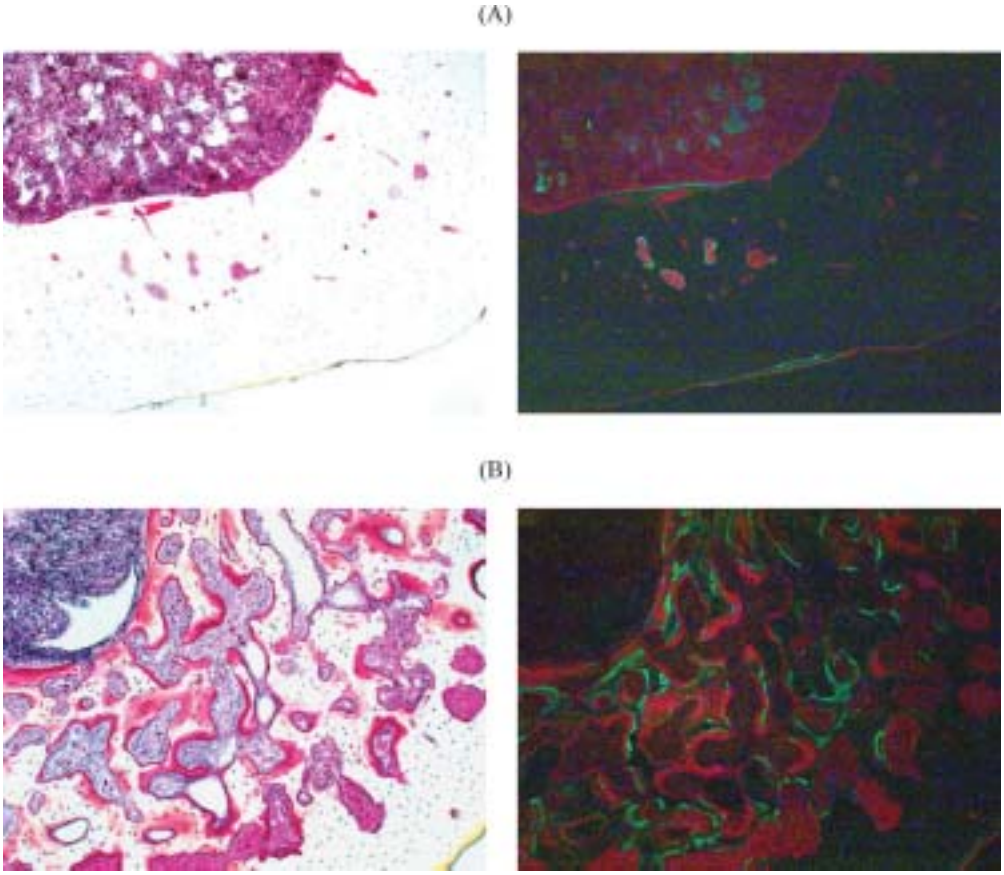

Fig. 5. Effects of DI-OCT + IV-OCT on bone histomorphometry. (**A**) DI-OCT + IV-OCT, (**B**) DI-vehicle + IV-OCT.

treatment. PDIT is a safe and effective treatment for advanced SHPT (Table 1). However, it has been suggested that the indication for PDIT is a patient who does not have a gigantic PTG (i.e. volume >2 cm^3) nor severely high levels of P and PTH (specifically, serum P and intact-PTH levels >9.0 mg/dL and >1500 pg/mL, respectively), and that patients with at least one of these criteria should be treated by PEIT [9]. Thus, the clinical indication of PDIT based on this limitation should be carefully discussed.

Conflict of interest statement. None declared.

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