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FAST TRACK

Nanogel-Based Delivery System Enhances PGE_2 Effects on Bone Formation

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Abstract Recovery of bone loss is one of the active research issues in bone medicine due to the need for efficient measures for bone gain. We examined here a novel drug delivery system using a nanogel of cholesterol-bearing pullulan (CHP) in combination with prostaglandin E_2 (PGE_2). PGE_2 or PGE_2/CHP, vehicle (saline containing 0.06% ethanol and 0.02% Tween 80) or CHP were injected on to the calvariae of mice once every day for 5 days per week for 4 weeks. Low dosage of PGE_2 (0.6 μg) alone or CHP alone did not induce new bone formation in this system. In contrast, PGE_2 (0.6 μg)/CHP induced new bone formation. Bone formation activities of PGE_2 was enhanced by CHP nanogels only at the site of injection (calvaria) but not in the distant sites of the skeleton, showing that PGE_2/CHP could avoid systemic effects. In spite of the fact that previously reported animal models of bone formation by PGE_2 were associated with loss of body weight, bone formation based on PGE_2/CHP did not associate with loss of body weight. Furthermore, only a single application of PGE_2 in combination with nanogel cross-linking hydrogel sphere (PGE_2/CHP-PEO) induced new bone formation. Thus, nanogel-based delivery system is an efficient delivery system of bone anabolic agent, PGE_2. J. Cell. Biochem. 101: 1063–1070, 2007.

Key words: drug delivery system; nanogel; cholesterol-bearing pullulan; prostaglandin E_2; bone formation

Repair of bone defects is one of the active research issues and development of technology to induce bone formation using biological reagents such as growth factors has been reported [Saito et al., 2001; Lutolf et al., 2003; Ito et al., 2005]. Since peptide growth factors are costly and their handling needs special care, non-peptydyl agents have advantages over peptide growth factors.

Prostaglandin E_2 (PGE_2) is a non-peptide anabolic agent for bone and its experimental efficacy has been shown in animals [Pilbeam et al., 2002]. However, PGE_2 at the dosages to induce bone formation causes severe diarrhea in the animals. Furthermore, due to the short half-life of PGE_2 in the circulation, PGE_2 treatment to induce bone formation in the previous animal models requires two to three

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times injections every day or continuous infusion for several weeks [Suponitzky and Weinreb, 1998; Keila et al., 2001; Yoshida et al., 2002]. Local injection of prostaglandin was also shown to be effective to obtain local formation of bone [Yang et al., 1993; Miller and Marks, 1994; Li et al., 2003]. Again in these experiments, daily injection is required and a particular from of delivery system was shown to be necessary. These features limit the application of PGE_2 as an anabolic agent for bone, while PGE_2 per se is safe and used for diseases in non-skeletal tissues. Although several drug delivery systems have been used for bone formation in experimental setting, most of them so far reported are those for BMP and peptide molecules.

Recent development of nano-science technology provides a break through in the control of bioactive molecules with regard to their holding and delivery in the target tissue. This is also enabling the possible novel design of drug delivery system in various tissues. Nanogel of cholesterol-bearing pullulan (CHP) and the nanogel-crosslinking materials have been developed as one of the nanocarrier systems for the drug including proteins or non-peptide molecules [Akiyoshi et al., 1993, 1996, 1998; Ikuta et al., 2002; Morimoto et al., 2005]. An amphiphilic hydrogel-matrix, consisting of cholesteryl group-associated hydrophobic domains and hydrophilic polysaccharide (polymer) chains in a CHP nanogel, provides effective drug-trapping sites in itself. Since PGE_2 is quickly inactivated when the molecule goes into circulation in the body, it is important to keep PGE_2 at the target site such as bone and to deliver for a certain period of time to accommodate with the speed of the bone formation which takes a relatively long time. Here, we investigated whether CHP nanogel would be useful to deliver PGE_2 for bone formation in vivo.

MATERIALS AND METHODS

Animals

Female ICR mice (3-week-old) were used for local injection experiments. Animals were killed after treatment for 4 weeks and both calvariae and femora were harvested. The mice were housed under controlled conditions at 24-h on a 12-h light/12-h dark cycle and fed with standard laboratory chow containing normal calcium and given tap water. Five mice were used for each experimental group or control. All animal experiments were approved by animal welfare committee of our university.

Experimental Protocol

Cholesterol-bearing pullulan (CHP) (Fig. 1A) was synthesized according to the previously published method [Akiyoshi et al., 1993, 1996, 1998; Ikuta et al., 2002]. Pullulan was substituted with 1.4 cholesterol moieties per 100

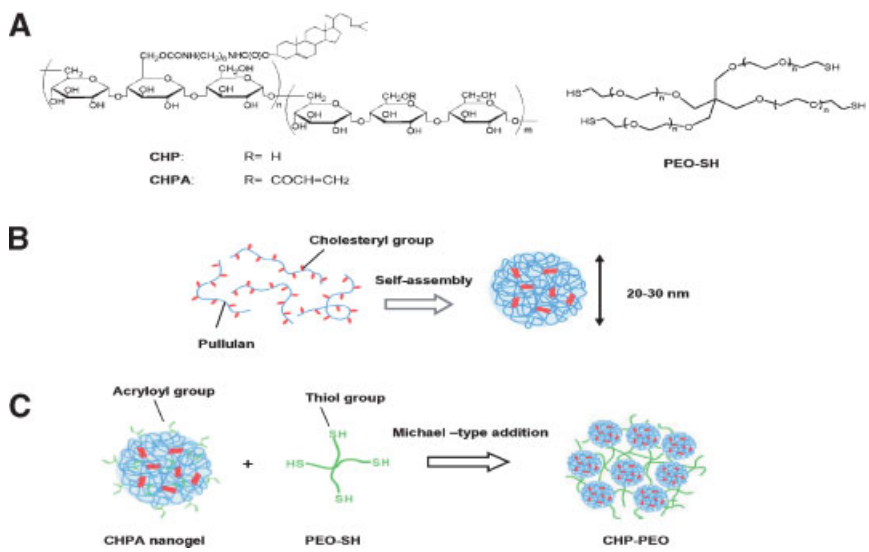

Fig. 1. Self-assembly of CHP nanogel and crosslinking based nanogel spheres (**A**) Chemical structure of CHP, CHPA and PEOSH; (**B**) formation of nanogel by self-assembly of CHP (**C**) Synthesis of CHP-PEO sphere based on Michael-type addition reaction. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

anhydrous glucoside units (Fig. 1A,B). CHP nanogel at 20 mg/ml was suspended in saline. PGE_2 (Wako Company, Tokyo, Japan) at 3 mg/ml was dissolved in saline supplemented with 3% ethanol and 1% Tween 80. PGE_2 solution was diluted with the solution containing CHP nanogel down to 60 μg/ml (i.e., 0.6 μg per 10 μl). This PGE_2/CHP solution was kept overnight at 4°C. For the local treatment, an aliquot (10 μl) of PGE_2 alone (0.6 μg), CHP alone, PGE_2 (0.6 μg)/CHP, or vehicle (saline containing 0.06% ethanol and 0.02% Tween80) was injected once a day for 5 days per week for 4 weeks onto the center of parietal bone of the mice.

CHP bearing acryloyl group (CHPA) (Fig. 1C) was synthesized by esterification mediated by dicyclohexylcarbodiimide (DCC). Hydrogen residue in CHP indicated in R in Figure 1A was substituted with acryloyl groups at the rate of 23 sites per 100 anhydrous glucoside units. CHPA nanogels containing PGE_2 (PGE_2/CHPA) were prepared in a same manner as PGE_2/CHP. Final concentration of CHPA nanogel was 25 mg/ml. Pentaerythritol tetra (mercaptoethyl) polyoxyethylene (PEOSH, Mw 9863, NOF Co.) was dissolved separately at 337 mg/ml in saline. CHPA nanogel in suspension and PEOSH solution were mixed at the volume ratio of 4:1, such that the ratio of thiol groups to acryloyl groups was 1:1. Then, 5 μl of the mixture was dropped onto Parafilm® and kept for 1 h at 37°C to obtain PGE_2/CHP-PEO gel sphere (2 mm in diameter). For the local treatment of bone with PGE_2 in CHP-PEO gel sphere, a single sphere was implanted onto the parietal bone of the calvaria in each mouse on the first day (only one single time implantation) and it was left for 4 weeks. To harvest calvariae, the mice were anesthetized with Avertin (tribromoethanol) and sacrificed.

Body Weight of the Animals

To monitor the effects of PGE_2 and CHP on the general body condition of these animals, body weight was measured everyday during the experimental periods. No major body weight alteration was observed in any experimental groups.

Micro-CT Analysis

The calvariae were subjected to 2D-μCT analysis using Musashi (Nittetsu-ELEX, Osaka, Japan) was evaluated, Tangential 2D-μCT pictures in frontal section plane of the parietal bone were used to measure PGE_2 effects on new bone. The thickness of calvariae was measured at five points between 1.26 and 3.78 mm (interval of 2.52 mm) away from the sagittal suture in the central region of parietal bone using frontal sections in microCT as described in part previously [Usui et al., 2002]. Averaged values of the calvarial thickness obtained from each mice (more than 5 mice per group) were used to calculate percent increase between the two groups to evaluate the response to the treatment. To examine the distant effects of local PGE_2 injection onto calvariae, bone volume in femora was evaluated. 2D-μCT slices were made within the midsagittal planes in the metaphysial cancellous bone region of femora. Bone volume/tissue volume (BV/TV) values were obtained in the 0.42 mm^2 square area (0.7 × 0.8 mm) located at 0.2 mm away from the growth plate of the distal end of femora. The image data were subsequently quantified using Luzex-F automated image analysis system (Nireco, Tokyo, Japan).

MTT and ALP Assay

Murine osteoblastic MC3T3E1 cells were seeded in 96-well plates (5×10^3 cells/well) and cultured over night in alpha-MEM supplemented with 5% FBS and antibiotics/antimycotics in a humidified atmosphere (5% CO_2–95% air). The cells were then cultured for 2 days in the presence of vehicle, CHP, 10 μM PGE_2, or CHP-10 μM PGE_2. At the end of the cultures, the cells were subjected to MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and ALP (alkaline phosphatase) assay as described elsewhere [Morinobu et al., 2005].

Statistical Analysis

Data were expressed as mean ± standard deviation and statistical evaluation was performed based on ANOVA using a statistical software package for Windows, Statview v.5.0 (SAS Institute). *P*-value less than 0.05 was considered to be statistically significant.

RESULTS

Although PGE_2 has been regarded as a bone anabolic agent, previous studies in our lab as well as others indicated that at least 0.6 μg PGE_2 should be injected "three times a day" onto the center of parietal bone and this should be continued every day for 4 weeks in order to

increase bone formation in mice [Yoshida et al., 2002; Tanaka et al., 2004; Sasaoka et al., 2004]. In contrast to the requirement for such multiple injections of PGE_2 alone, only "once a day" injection of PGE_2 in CHP nanogels increased the thickness of the calvariae of mice (Fig. 2A, PGE_2/CHP vs. Cont). As a control, we injected 0.6 μg of PGE_2 without CHP nanogels "once a day" onto the animals. "Once a day" injection of 0.6 μg of PGE_2 alone without CHP did not increase the thickness of the calvariae as known previously (Fig. 2A, PGE_2 vs. Cont). CHP nanogels alone without PGE_2 did not alter calvariae thickness either (Fig. 2A, CHP vs. Cont).

Quantification of the thickness of calvariae to evaluate the response to PGE_2 indicated no increase (percent increase) in the thickness (Fig. 2B, left column, control vs. PGE_2) of the calvariae by PGE_2 injection alone compared to control. In contrast, calvarial bone responded to PGE_2 administration in combination with CHP nanogel and increased thickness of the calvaria more than CHP alone (Fig. 2B, PGE_2/CHP vs. CHP).

One of the major difficulties in the treatment with PGE_2 for the purpose of the bone formation was that systemic injection of PGE_2 in animals caused severe diarrhea and loss of body weight even when the PGE_2 dosage was minimally high enough for bone formation [Jee and Ma, 1997; Suponitzky and Weinreb, 1998]. We, therefore, examined whether the injection protocol used in our experiments in Figure 2 may affect body weight. No change in body weight was observed in the mice treated with single 0.6 μg PGE_2 injection with or without CHP (data not shown). Another interest for us was to see whether PGE_2 effects on bone were localized to the site of injection or whether they could be seen in other part of the body. To check this point, we quantified the cancellous bone mass levels in distal femur of the mice where the PGE_2/CHP was injected onto calvariae. Even when single injection with 0.6 μg PGE_2 alone (without CHP) onto calvariae did not enhance the thickness of the calvariae, such administration protocol with single PGE_2 (0.6 μg) injection (without) CHP at calvaria resulted in a minor increase in the cancellous bone mass in the femur (Fig. 3A,B).

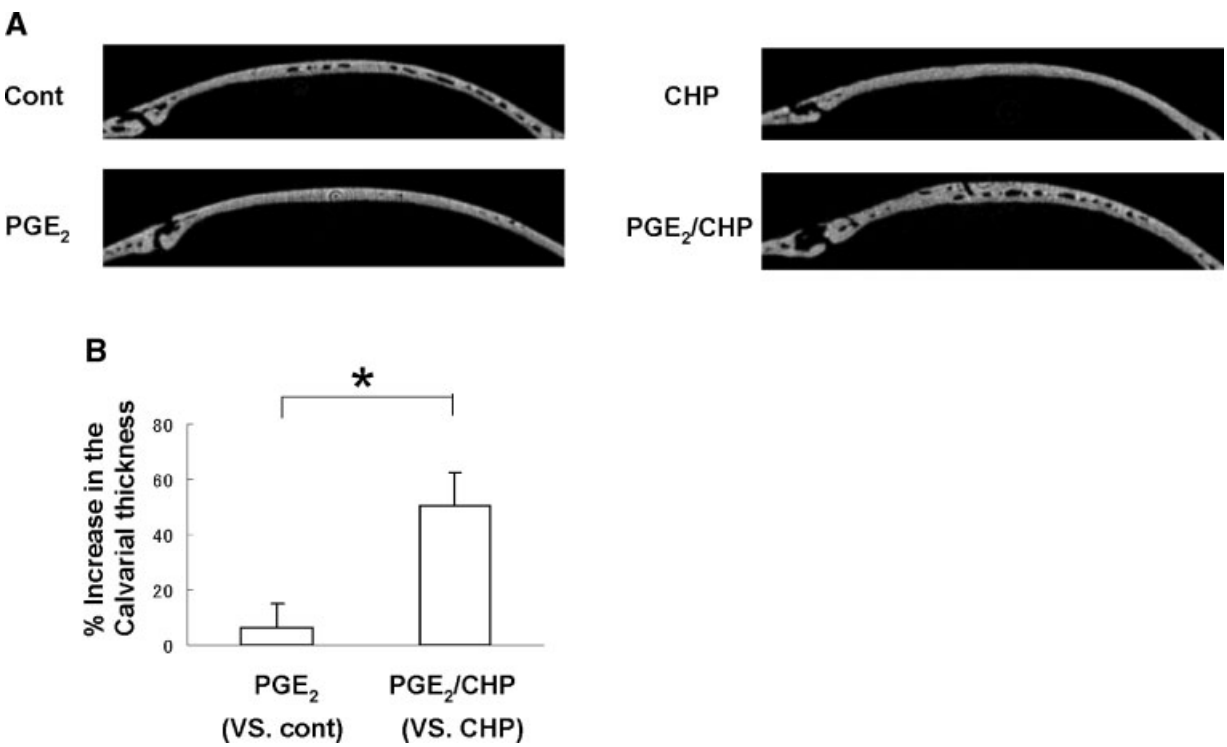

Fig. 2. Nanogel-based PGE_2 delivery increased clvarial bone mass. **A**: Micro-CT pictures showing frontal cross section of calvarial (parietal) bone of mice subjected to ''one injection per day'' protocol of PGE_2/CHP (or vehicle). **B**: Calvarial thickness was measured in the frontal cross section as described in Material and Methods and expressed as ratio.

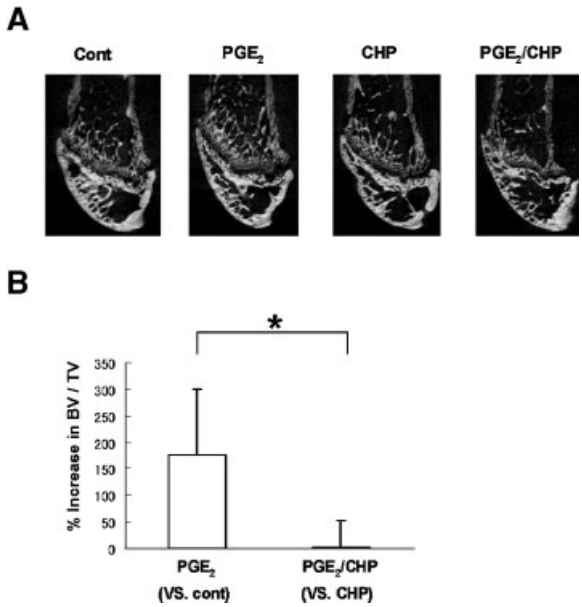

Fig. 3. Nanogel-based delivery did not affect bone mass in bone distant from injection sites. **A**: μCT pictures of distal ends of femora from mice after PGE_2/CHP treatment in their calvaria. **B**: Cancellous bone in the distal femora of PGE_2/CHP treated mice was subjected to quantification for bone volume per tissue volume (BV/TV) and PGE_2 effects are indicated as ratio over control.

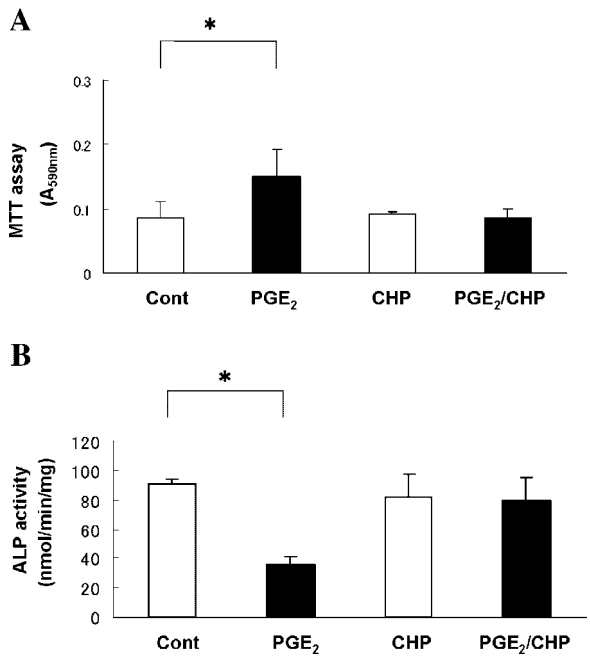

Fig. 4. PGE_2/CHP did not release PGE_2 it into medium in vitro. Proliferation (MTT assay) (**A**) and differentiation (alkaline phosphatase assay) (**B**) in osteoblastic cells in the presence of PGE_2/CHP or PGE_2 in medium for 2 days were evaluated as described in materials and methods.

This is possibly due to the higher sensitivity to PGE_2 in cancellous bone compared to calvarial cortical bone. In contrast to such distant effects of single injection of "CHP-free" PGE_2 onto the calvariae, PGE_2/CHP injection did not show any change in cancellous bone mass in the femur even when the PGE_2/CHP injection onto calvaria increased the thickness of the calvaria which is basically a cortical bone (Fig. 3A,B). CHP injection alone did neither affect cancellous bone mass in the femur (Fig. 3A). Thus, CHP/PGE_2 injection targeted bone formation at the site of injection and avoided in distant sites is influence.

Since we did not observe any distant effect of a PGE_2 when PGE_2/CHP was injected into calvariae, we hypothesized that PGE_2 was trapped into the CHP and it was released from the nanogel locally. This could prevent fast diffusion into the whole body. In order to test this point, we examined the property of the nanogels to trap PGE_2 by cell proliferation assay using osteoblast-like MC3T3E1 cells. When PGE_2 alone was added to the medium, MTT assay indicated the increase in proliferation of the osteoblastic cells (Fig. 4A). In contrast, although the same amount of PGE_2 in CHP was delivered into the medium, PGE_2/CHP did not influence cells at least for the period of 2 days during the assay (Fig. 4A). With respect to PGE_2 effect on osteoblastic differentiation, alkaline phosphatase activity was examined. Single PGE_2 addition (without CHP nanogel) into the medium suppressed the expression of alkaline phosphatase activity in osteoblastic cells (Fig. 4B). However, PGE_2/CHP did not suppress alkaline phosphatase expression in osteoblastic cells (Fig. 4B). Thus, PGE_2/CHP nanogels prevented at least these responses of the cells in culture to PGE_2.

As bone formation is a long-time process which takes several weeks rather than days, we further wished to establish a new design of PGE_2/CHP application for bone formation to enable "single injection" per "4 weeks" rather than "single injection" every day. To do this, we designed PGE_2/CHP-PEO. This system contains nanogel-crosslinking hydrogel spheres (CHP-PEO) where acryloyl group-bearing CHP (CHPA) nanogels containing PGE_2 were cross-linked with pentaerythritol tetra (mercaptoethyl) polyoxyethylene (PEO-SH) (Fig. 1A,C). These CHPA nanogels are useful for building blocks and multiple cross-linkers to

construct shaped hydrogel materials. PGE_2/CHP-PEO would stay for longer time period at the site of implantation due to the slow diffusion of the nanogels in a form of the cross-linking PEO matrix. We have tested whether "once per 4 weeks" injection of varying amounts of PGE_2. PGE_2 was incorporated into CHP-PEO hydrogel spheres (diameter, 2 mm) to see whether they may alter the responses in the calvarial bone. The result indicated that only one single implantation of 0.6 μg PGE_2 within CHP-PEO gel spheres increased the thickness of calvariae(Fig. 5A). Examination of bone mass at sites distant from injection such as femur indicated that single application of PGE_2/CHP-PEO per 4 weeks did not alter bone volume (Fig. 5C,D). When the dosage was increased up to 6 and 12 μg (Fig. 5B), the PGE_2/CHP-PEO increased the thickness of calvaria similarly in these dosages tested. In contrast to calvariae, bone in distant sites, such as cancellous bone in the femora, did not respond to PGE_2 at any of the higher dosages (Fig. 5C,D). The similar anabolic effects of PGE_2/CHP-PEO on the calvarial bone thickness among the dosages from 0.6, 6, and 12 μg indicate the high capacity of CHP-PEO to hold PGE_2. Again no alterations in body weight was observed in the mice treated with any dosage of PGE_2/CHP-PEO, which were injected onto parietal bone (data not shown).

DISCUSSION

Our observations indicated that PGE_2/CHP could efficaciously deliver the non-peptide bone anabolic agents, PGE_2, to increase bone at targeted sites without having any side effects in the animals. By using cross-linked nanogel, CHP-PEO, relatively low levels (0.6 μg) of PGE_2 in PGE_2/CHP was shown to provide efficient bone formation even via "a single application per 4 weeks," indicating the efficacy of this drug delivery system without otherwise inevitable inactivation of prostaglandin in the lung during systemic circulation.

Bone formation is required in a number of cases of adult bone defects such as major fractures, tumors, injuries, or revision surgeries of artificial joints. Although PGE_2 has been proven to be stimulatory for bone formation in several types of experimental models in vivo, it has not yet been clinically used for repair of bone defects due to their relatively low efficacy per single injection in vivo. Another problem associated with PGE_2 is the significant side effects of PGE_2 in animal models of bone formation. When animals were treated with three times injection with PGE_2 per day for 4 weeks in order to obtain anabolic effects on bone, diarrhea, and loss of body weight associated with the bone change.

PGE_2 is labile due to immediate inactivation after one circulation in lung. PGE_2 has been already proven to be efficacious to induce bone formation in both human and animals [Pilbeam et al., 2002]. The problem with PGE_2 is its short half-life in the circulation and its multiple administrations per day. We showed that CHP nanogel has solved such problems based on the efficient trapping of PGE_2 in the nanogel to avoid the multiple injections per day and at the same time to avoid its systemic side effects. Nanogel delivery may also prevent inactivation of PGE_2 due to its release into circulation.

In addition to PGE_2/CHP, which reduced the number of injection per day from three times to once, we also developed PGE_2/CHPA delivery system which requires only once per 4 weeks to enhance bone mass. This provides a major advantage to avoid daily injection per se. For further application of PGE_2, reduction of injection procedures would avoid the possibilities for infection and would also contribute the cost-effectiveness.

Importantly, CHP has been proven to be safe in human and is absorbable in vivo. The strength of nanogel is not simply to hold the drug such as PGE_2 but its long term effects seen in the increase in the thickness of calvariae bone. This is an advantage over the other drug careers such as polylactic acid, which can attach PGE_2 but does not hold the drug for a long time to enable slow release.

In conclusion, PGE_2/CHP and PGE_2/CHP-PEO are a novel efficacious delivery system for the treatment of bone defects.

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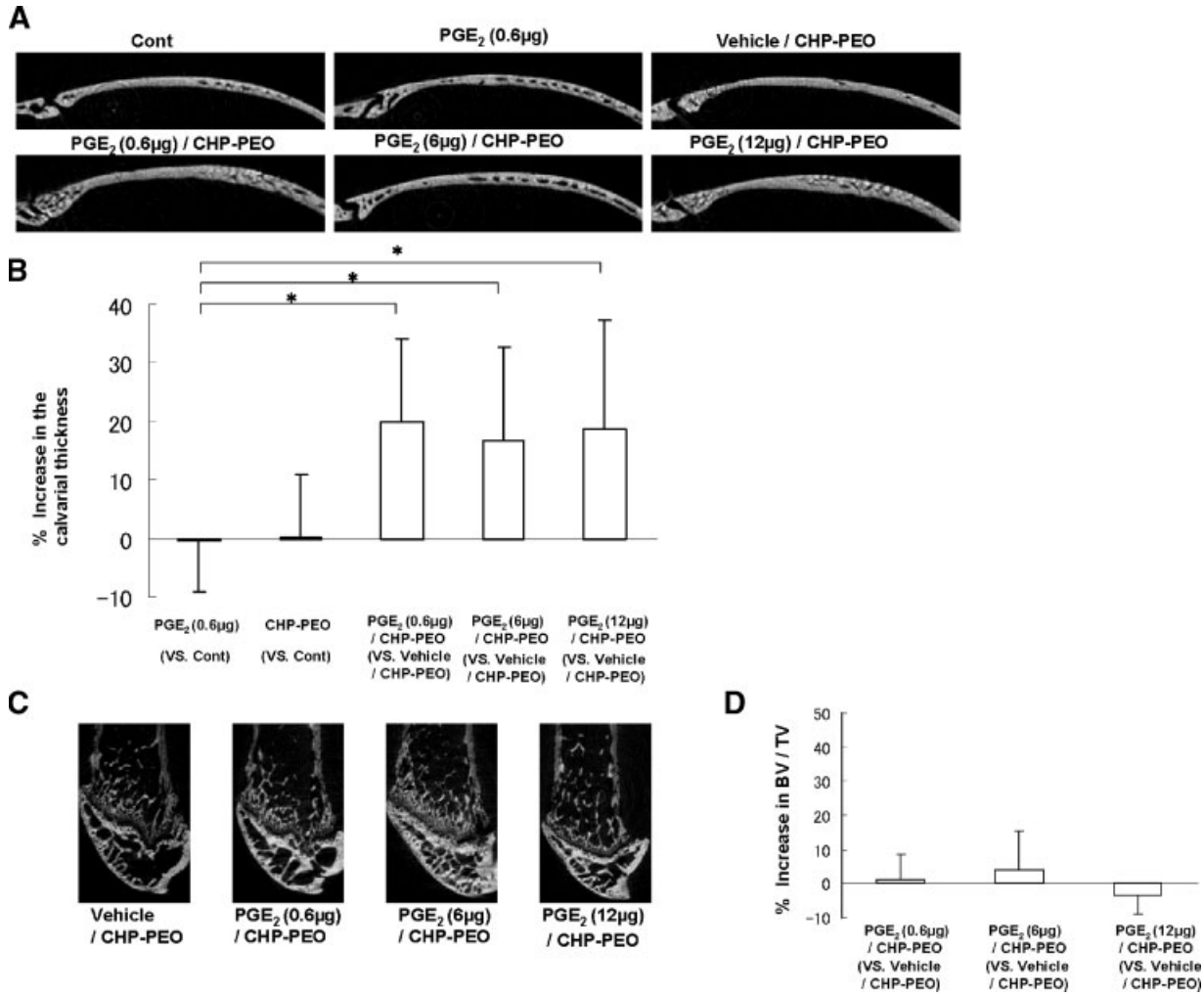

Fig. 5. Nanogel-based PGE_2 delivery via crosslinking gel exhibited bone mass enhancement after ''once per 4 weeks'' treatment. **A**: Micro-CT pictures of the calvariae of mice after ''once per 4 weeks'' treatment with PGE_2/CHP-PEO (or vehicle). **B**: Thickness in calvaria shown in (A) was measured as described in material and methods and expressed as % increase relative to control. **C**: μCT pictures of the distal end of femora from mice after PGE_2/CHP-PEO treatment in their calvariae. **D**: Cancellous bone in the distal end of femora of PGE_2/CHP-PEO gel-treated mice was quantified and shown as % increase in bone volume per tissue volume (BV/TV).

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