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Original article

Collagen-binding peptide reverses bone loss in a mouse model of cerebral palsy based on clinical databases

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ABSTRACT

Background: Individuals with cerebral palsy (CP) experience bone loss due to impaired weight bearing. Despite serious complications, there is no standard medication.
Objective: To develop a new pharmacological agent, we performed a series of studies. The primary aim was to develop an animal model of CP to use our target medication based on transcriptome analysis of individuals with CP. The secondary aim was to show the therapeutic capability of collagen-binding peptide (CBP) in reversing bone loss in the CP mouse model.
Methods: A total of 119 people with CP and 13 healthy adults participated in the study and 140 mice were used for the behavioral analysis and discovery of therapeutic effects in the preclinical study. The mouse model of CP was induced by hypoxic-ischemic brain injury. Inclusion and exclusion criteria were established for CBP medication in the CP mouse model with bone loss.
Results: On the basis of clinical outcomes showing insufficient mechanical loading from non-ambulatory function and that underweight mainly affects bone loss in adults with CP, we developed a mouse model of CP with bone loss. Injury severity and body weight mainly affected bone loss in the CP mouse model. Transcriptome analysis showed *SPP1* expression downregulated in adults with CP who showed lower bone density than healthy controls. Therefore, a synthesized CBP was administered to the mouse model. Trabecular thickness, total collagen and bone turnover activity increased with CBP treatment as compared with the saline control. Immunohistochemistry showed increased immunoreactivity of runt-related transcription factor 2 and osteocalcin, so the CBP participated in osteoblast differentiation.
Conclusions: This study can provide a scientific basis for a promising translational approach for developing new anabolic CBP medication to treat bone loss in individuals with CP.

1. Introduction

Although the primary consequences of cerebral palsy (CP) are associated with brain insults, people with CP who experience impaired physical activity often experience musculoskeletal problems [1,2], particularly bone loss in adulthood owing to accumulative paralysis and impaired weight bearing resulting from mechanical adaptations [3,4]. Bone loss in adults with CP probably occurs at an early age, thus leading to aggravated symptoms as problems accumulate into adulthood [5]. Eventually, adults with CP commonly experience atypical fragility fractures and unfavourable prognoses such as malunion after musculoskeletal surgeries [3,6].

Despite the serious complications, a standard therapy has not been established for bone loss in CP [3]. Pharmacological interventions such as bisphosphonate and intermittent exposure to parathyroid hormone have been introduced to manage bone health in CP [7,8]. However, therapeutic mechanisms are inappropriate as lifelong medications in CP because those indications are more specific for osteogenesis imperfecta [9,10], postmenopausal osteoporosis, and senile osteoporosis [11–13]. Therefore, a novel

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anabolic therapy should be developed and optimized for people with CP [14].

To develop a new pharmacological agent and therapeutic strategy, we performed a series of translational studies. First, significant clinical factors affecting bone health were identified in individuals with CP, then transcriptome analysis was conducted to identify imbalanced gene expression in people with CP as compared with normal controls. Second, a mouse model of CP with bone loss was developed by using modified neonatal hypoxic-ischemic (HI) brain-injured mice based on the preceding clinical outcomes. Finally, from transcriptome analyses, a novel treatment using synthesized collagen-binding peptide (CBP) of osteopontin was administered to treat the mouse model of CP with bone loss. The research hypothesis was that a complex interplay of severe brain injury, low body weight, insufficient mechanical loading, and reduced muscle force affects overall bone health in CP. The clinical relevance of administering CBP might be determined by mimicking human-like features in an animal model of CP with bone loss. This study could provide a scientific basis for a promising translational approach to treating bone loss in people with CP.

2. Materials and methods

2.1. Study approval

This study was approved by the Institutional Review Board (No. 4-2012-0751; 4-2013-0404) of Yonsei University Health System. Informed consent was acquired from all participants, who agreed to the procedures in the prospective study. All research procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (IACUC No. 2016-0019; 2018-0277) [15].

2.2. Clinical outcome variables in patients with CP

A total of 125 patients from our hospital were recruited from 2007 to 2015 for the present retrospective study. Six patients were excluded for reasons such as missing height and femur bone density data. In total, 47 patients with missing data including functional outcomes were excluded from the regression analyses. Finally, 72 outcome variables including age, body weight, Modified Barthel Index (MBI), grip strength, and ambulatory function were used for regression analysis. Linear regression analysis of the clinical parameters was used to find the most significant clinical risk factors associated with bone health. The MBI was used to identify functional level from activities of daily living. Grip strength was used to identify the maximal force when holding a dynamometer.

To identify regional BMD based on ambulatory status and body weight classified by body mass index (BMI), 4 groups of CP participants were distinguished: ambulatory and normal weight ($n = 55$), ambulatory and underweight ($n = 8$), non-ambulatory and normal weight ($n = 39$), and non-ambulatory and underweight ($n = 17$). Participants were also classified based on ambulatory status (ambulatory and non-ambulatory) and CP type (spastic and dyskinetic) determined as in a previous study [3].

2.3. Participants in transcriptome analysis

To propose an optimized bone anabolic agent for people with CP, pathophysiological factors imbalanced in individuals with CP versus healthy adults were analysed by transcriptome analysis. From 2013 to 2015, 26 participants (13 with CP and 13 healthy adults) agreed to participate in gene expression screening at the mRNA level as a prospective analysis (Table 3). The number of individuals in the transcriptomic analysis could not be matched with regression analysis ($n = 72$) owing to agreement with the prospective study and sample use. After screening medical records, participants who were ineligible in this study were excluded based on previously described criteria: age < 20 years; previous medication affecting bone metabolism within 1 year, such as anticonvulsant, bisphosphonate, glucocorticoid; any illness affecting bone metabolism, such as bone tumor, hyperparathyroidism, Cushing's syndrome, and rheumatoid arthritis; and systemic disease such as diabetes, kidney, liver and thyroid disease [3]. Clinical characteristics of participants with CP and age-matched healthy controls included are in Table 3.

2.4. Neonatal hypoxic-ischemic (HI) brain injury

To develop an animal model mimicking features of individuals with CP, we used a modified Rice–Vannuci HI model [16]. By using a ventral neck incision and exposure of the right common carotid artery at the mid-tracheal level, 7-day-old CD-1 mice underwent permanent ischemic brain injury induced by unilateral ligation of the common carotid artery under anaesthesia with isoflurane. After a 2-hr recovery, mice underwent 8% O_2 hypoxic conditions for 90 min in a chamber maintained at 37.5 °C. The mice then returned to their dams under normal air conditions [15].

2.5. Inclusion and exclusion criteria for therapeutic application

The inclusion and exclusion criteria were established to identify the effects of the CBP treatment on mice with bone loss after neonatal HI brain injury. The patent number is 10-2019-0016496 (Korea). Two main factors were considered based on linear regression analyses, severity of the brain injury and body weight. HI brain-injured mice at age 2 weeks were classified as having severe and mild HI and mild brain-injured mice were excluded. Mice with body weight < 24.4 g for females and < 31.5 g for males at 6 weeks of age were included, and mice with body weight within the normal range were excluded. Finally, animals regularly showing hyperactive behaviour such as turning around or rearing repetitively, were excluded from the therapeutic timeline (Fig. 2A–C) [17].

2.6. Synthesis of the CBP

Osteopontin residues 150–168 had the strongest binding affinity to collagen. The sequence was synthesized with a peptide synthesizer (Apex 396; AAPP Tec, Louisville, KY, USA) from a pharmaceutical firm (NIBEC, Seoul, Korea) as described [18,19]. The purity of the peptides was confirmed to be $> 98\%$ (Fig. 2D).

2.7. Therapeutic strategy in an animal model of CP

Neonatal HI brain-injured mice with bone loss (severe brain injury $\times$ low body weight) were included in accordance with the inclusion and exclusion criteria. From these criteria, mice were randomly allocated to CBP treatment or saline control. The CBP treatment group was subcutaneously injected with a high concentration of CBP peptides (600 μg per mouse) biweekly for 2 months beginning at 8 weeks of age (Fig. 2C) [19]. Other procedures are described in the Online material.

2.8. Statistical analysis

All statistical analyses were performed with SPSS Statistics 23 (IBM Corp., Armonk, NY, USA). Regression analysis was performed

to determine relationships between multiple factors and BMD. Stepwise multivariate analysis was performed to investigate factors associated with regional BMD. Chi^2 test was used to compare 2 groups. ANOVA was followed by Fisher's LSD *post hoc* test for comparing ≥ 3 groups. A two-way ANOVA with one repeated measure was used to test the interaction between time and groups (6×3 mixed design), as well as the main effect between groups. The time effect was not considered in this study because bone growth in rodents continues after sexual maturity [20]. An independent *t*-test was used to compare the difference between 2 groups. $P < 0.05$ was considered statistically significant.

An *a priori* analysis was used to estimate sample size with the effect size based on a previous study [21], showing a medium-to-large effect size in postmenopausal women: 58 participants were required to provide a minimum of 80% statistical power to detect factors associated with BMD on linear multiple regression analysis. With a very large effect size (approximately $f^2 = 1.0$) in mice in this study, we needed 20 mice to provide a minimum of 80% statistical power.

3. Results

3.1. Risk factors associated with bone health in individuals with CP

The mean (SD) age of the 72 individuals with CP was 39.5 (11.1) years, height was 158.8 (6.5) cm, weight 55.0 (9.8) kg, and BMI 21.8 (3.7) kg/m^2. Linear regression analysis to find the most significant clinical risk factors associated with bone loss in individuals with CP revealed ambulatory function as the most influential factor associated with lumbar spine, femur intertrochanter, and total hip BMD. Body weight was also a significant factor (Table 1).

3.2. Bone density based on ambulatory status and body weight

Clinical characteristics of 119 participants with CP are shown in Table 2. The 4 groups differed in lumbar spine BMD (Z-scores; $F_{(3,\ 115)} = 3.870$; $P = 0.011$), femur neck BMD ($F_{(3,\ 115)} = 7.615$; $P < 0.001$), femur intertrochanter BMD ($F_{(3,\ 115)} = 12.641$;

Table 1
Clinical factors associated with skeletal bone mineral density (BMD) in individuals with cerebral palsy (CP) ($n = 72$).

BMD region	Variables (reference)	Univariate analysis		Multivariate analysis 2nd model		Multivariate analysis Final model	
		Coefficient (SE)	*P*-value	Coefficient (SE)	*P*-value	Coefficient (SE)	*P*-value
Lumbar spine	Sex (female)	−0.361 (0.279)	0.201	–	–	–	–
	Body weight	0.025 (0.014)	0.089	–	–	–	–
	Modified Barthel Index	0.012 (0.005)	0.030*	–	–	–	–
	Grip strength	0.010 (0.005)	0.028*	–	–	–	–
	Ambulation (non-ambulator)	0.818 (0.270)	0.003*	–	–	0.818 (0.270)	0.003*
Femur neck	Sex (female)	−0.079 (0.263)	0.764	–	–	–	–
	Body weight	0.036 (0.013)	0.007*	0.032 (0.012)	0.012*	–	–
	Modified Barthel Index	0.015 (0.005)	0.004*	0.013 (0.005)	0.007*	0.015 (0.005)	0.004*
	Grip strength	0.007 (0.004)	0.126	–	–	–	–
	Ambulation (non-ambulator)	0.649 (0.256)	0.013*	–	–	–	–
Femur intertrochanter	Sex (female)	−0.420 (0.266)	0.119	–	–	–	–
	Body weight	0.030 (0.014)	0.032*	0.027 (0.013)	0.033*	–	–
	Modified Barthel Index	0.017 (0.005)	0.001*	–	–	–	–
	Grip strength	0.012 (0.004)	0.006*	–	–	–	–
	Ambulation (non-ambulator)	0.894 (0.253)	0.001*	0.866 (0.247)	0.001*	0.894 (0.253)	0.001*
Total hip	Sex (female)	−0.374 (0.264)	0.161	–	–	–	–
	Body weight	0.035 (0.013)	0.010*	0.033 (0.012)	0.010*	–	–
	Modified Barthel Index	0.016 (0.005)	0.002*	–	–	–	–
	Grip strength	0.012 (0.004)	0.009*	–	–	–	–
	Ambulation (non-ambulator)	0.823 (0.253)	0.002*	0.790 (0.244)	0.002*	0.823 (0.253)	0.002*

Grip strength (percentage normalized based on age- and sex-matched strength of healthy adults); SE: standard error; $R^2 = 0.116$, adjusted $R^2 = 0.104$ in the analysis of the lumbar spine BMD; $R^2 = 0.110$, adjusted $R^2 = 0.098$ for femur neck BMD; $R^2 = 0.151$, adjusted $R^2 = 0.139$ for femur intertrochanter BMD; $R^2 = 0.131$, adjusted $R^2 = 0.118$ for total hip BMD; *: $P < 0.05$ based on linear regression analyses.

Table 2
Demographic and clinical parameters in the clinical study by categories of CP ($n = 119$).

Variables (unit)	Ambulatory and normal weight CP ($n = 55$)	Ambulatory and underweight CP ($n = 8$)	Non-ambulatory and normal weight CP ($n = 39$)	Non-ambulatory and underweight CP ($n = 17$)
Age (years)	39.4 (11.2)	39.5 (8.8)	39.0 (10.7)	36.9 (12.8)
Height (cm)	159.4 (7.6)	158.1 (8.5)	158.5 (7.4)	159.7 (8.9)
Weight (kg)	57.5 (10.0)	42.6 (6.8)†	56.6 (6.3)	41.6 (5.5)*
Body mass index (kg/m^2)	22.6 (3.2)	17.0 (1.2)†	22.6 (2.4)	16.4 (2.0)*
Sex (male/female)	28/27	2/6	23/16	9/8
Type (spastic/dyskinetic)	19/36	1/7	22/17	7/10
Bone status§ (normal/osteopenia/osteoporosis)	26/23/6	3/5/0	6/20/13	1/4/12

Data are mean (SD). *: $P < 0.05$ comparing non-ambulatory and underweight vs. non-ambulatory and normal weight; non-ambulatory and underweight vs. ambulatory and normal weight); †: $P < 0.05$ comparing ambulatory and underweight vs. non-ambulatory and normal weight; ambulatory and underweight vs. ambulatory and normal weight based on *post hoc* comparison after one-way ANOVA. §: $P < 0.05$ based on Chi^2 test.

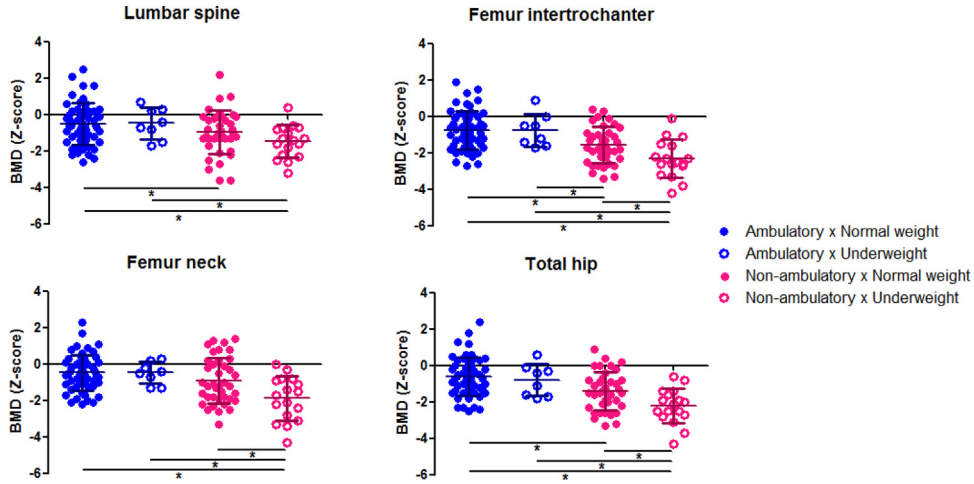

Fig. 1. Significant differences in bone mineral density (BMD) of the lumbar spine and femur based on ambulatory status and body weight of individuals with cerebral palsy (CP). Sample size (ambulatory and normal weight CP, $n = 55$; ambulatory and underweight CP, $n = 8$; non-ambulatory and normal weight CP, $n = 39$; non-ambulatory and underweight CP, $n = 17$); data are mean ± SD; *: $P < 0.05$ based on *post hoc* comparison after one-way ANOVA.

$P < 0.001$) and total hip BMD ($F_{(3,\ 115)} = 12.058$; $P < 0.001$). Statistical outcomes are shown in Fig. 1.

3.3. The top-ranked gene based on transcriptome analysis

The transcriptome database was used to identify dysregulated gene expression associated with bone metabolism. BMD z-scores for the femur neck and total hip were lower for participants with CP than healthy controls (Table 3). Finally, the focus was on the top-ranked gene, secreted phosphoprotein 1 (*SPP1*), which was significantly downregulated, showing 70.1-fold change in mRNA level in individuals with CP versus healthy adults (Online material Table 1).

3.4. Impaired muscle force in an animal model of CP

Contralateral forelimb analysis showed significantly decreased grip strength in severely brain-injured mice versus both mildly brain-injured mice and normal mice ($F_{(2,\ 123)} = 28.733$; $P < 0.001$, *post hoc* comparison after one-way ANOVA; Fig. 2E).

3.5. Linear regression analysis of factors critical for bone loss

From univariate analysis of whole-skeletal BMD, the severity of brain injury, body weight, contralateral forelimb grip strength, and the number of rearing instances were significantly correlated in the CP model. The results for lumbar spine, affected femur and humerus BMD are in Table 4. In the final stepwise model, severity of brain injury was the most influential factor for both whole-skeletal and lumbar-spine BMD. Body weight was associated the most with both affected femur and humerus BMD.

Table 3
Baseline characteristics of participants in transcriptome analyses.

Baseline character (unit)	Healthy adults ($n = 13$)	Participants with CP ($n = 13$)
Age (years)	34.4 (7.3)	39.5 (8.0)
Height (cm)	168.3 (8.8)	159.0 (7.7)*
Weight (kg)	60.5 (13.4)	55.8 (8.7)
Body mass index (kg/m^2)	21.2 (3.2)	22.2 (3.4)
[underweight/normal/overweight (n)]	[1/10/2]	[2/10/1]
Sex [male/female (n)]	4/9	6/7
Type [spastic/dyskinetic (n)]	NA	6/7
Ambulatory status [independent/wheelchair (n)]	13/0	7/6
Bone status [normal/osteopenia/osteoporosis (n)]	13/0/0	3/6/4
Lumbar spine BMD (z-score)	0.3 (0.7)	−0.5 (1.6)
Femur neck BMD (z-score)	0.2 (0.5)	−0.7 (1.1)*
Total hip BMD (z-score)	0.6 (1.1)	−1.2 (0.9)*

Data are mean (SD). NA: not associated; *: $P < 0.05$ based on independent t-test.

3.6. Bone loss in mice with neonatal HI brain injury

From the criteria established by linear regression analyses, severely brain-injured mice with low body weight showed the lowest skeletal BMD in whole-body skeleton, lumbar spine from L1–L5, affected femur and affected humerus as compared with mild or severely brain-injured mice with normal body weight in adulthood.

Analysis of the whole-skeletal BMD revealed no significant time × group interaction (Wilk's lambda = 0.745; $F_{(10,\ 78)} = 1.234$; $P = 0.283$). The main effect of group ($F_{(2,\ 43)} = 13.715$; $P < 0.001$) was observed by two-way repeated-measures ANOVA (Fig. 2F). Analysis of the lumbar spine BMD revealed no significant time × group interaction (Wilk's lambda = 0.576; $F_{(10,\ 78)} = 2.477$; $P = 0.124$). The main effect of group ($F_{(2,\ 43)} = 10.565$; $P < 0.001$) was significant.

Analysis of the affected femur BMD revealed no significant time × group interaction (Wilk's lambda = 0.924; $F_{(10,\ 78)} = 0.316$; $P = 0.975$). The main effect of group ($F_{(2,\ 43)} = 8.818$; $P = 0.001$) was significant. Similarly, analysis of the affected humerus BMD revealed no significant time × group interaction (Wilk's lambda = 0.819; $F_{(10,\ 78)} = 0.821$; $P = 0.609$). The main effect of group ($F_{(2,\ 43)} = 24.888$; $P < 0.001$) was significant.

3.7. Effect of CBP treatment on bone loss

A representative 3-D image of skeletal bone showed trabecular bone density improved in the CBP treatment group versus the saline control. Micro-CT (μCT) analysis of skeletal bone confirmed that trabecular thickness in the femora ($P = 0.023$, independent t-test) and humeri ($P = 0.040$) of CBP-treated mice was significantly increased as compared with the saline control (Fig. 3A and B). Total collagen content was significantly improved in the cortical- and trabecular-containing region in the CBP treatment group versus the saline control group ($F_{(2,15)} = 9.036$; $P = 0.003$, *post hoc* comparison after one-way ANOVA; Fig. 3C and D).

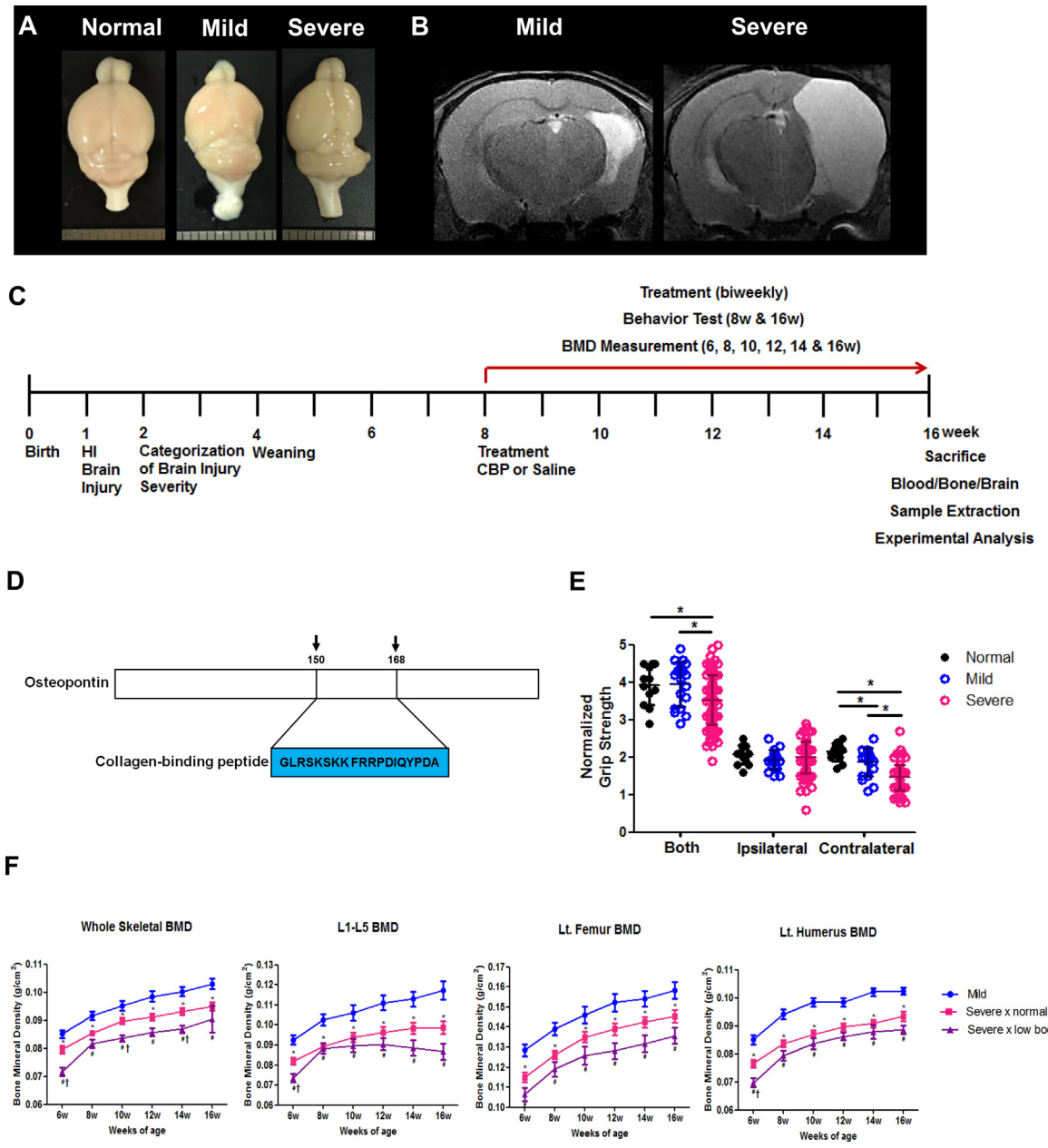

Figure 2. Experimental timeline for therapeutic intervention in an animal model of CP with bone loss. A. Post-mortem assessment to confirm injury severity. B. Determined infarct size and comparison of lesion locations between mild brain injuries and severe brain injuries on MRI. C. Summarized experimental procedures from birth to 16 weeks of age. D. Sequences of the collagen-binding peptide (CBP) of osteopontin. E. Hemiplegic motor impairments in severely brain-injured mice. F. Bone loss in severely brain-injured mice with low body weight. A statistically significant group effect was confirmed ($P < 0.05$) based on a two-way repeated measures ANOVA in whole body skeleton, lumbar spine, affected femur, and affected humerus. Data are mean ± SD. *E.* Sample size (normal, $n = 12$; mild brain injury, $n = 18$; severe brain injury, $n = 96$); *: $P < 0.05$ based on *post hoc* comparison after one-way ANOVA. *F.* Sample size (mild brain injury, $n = 18$; severe brain injury × normal body weight, $n = 22$; severe brain injury × low body weight, $n = 6$); *: $P < 0.05$ comparing mild brain injury vs. severe brain injury × normal body weight; #: $P < 0.05$ comparing mild brain injury vs. severe brain injury × low body weight; †: $P < 0.05$ comparing severe brain injury × normal body weight vs. severe brain injury × low body weight based on *post hoc* comparison after one-way ANOVA. HI: hypoxic-ischemic; grip strength normalized to body weight (g/g).

3.8. Effect of CBP treatment on bone turnover

Mice were sacrificed and serum samples were used to identify the effect of CBP on bone turnover activity. Both PINP ($P = 0.018$, independent *t*-test) and CTX-I ($P = 0.020$) were highly upregulated in the CBP treatment group versus the saline control group (Fig. 3E and F).

3.9. Effect of CBP treatment on osteoblast differentiation

To determine the role of CBP in severely brain-injured mice with bone loss, markers associated with osteoblast differentiation, including runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN), were detected on immunohistochemistry. The expression of RUNX2 was higher in the CBP treatment than saline control group ($P = 0.005$, independent *t*-test; Fig. 3G and H). The expression of OCN was also higher in the CBP treatment than saline control group ($P = 0.001$; Fig. 3I and J).

4. Discussion

From clinical discovery, insufficient mechanical loading affected by non-ambulatory function and low body weight were the

Table 4
Factors associated with skeletal BMD in a mouse model of CP (n = 24).

BMD region	Variables (reference)	Univariate analysis		Multivariate analysis	
		Coefficient (SE)	*P*-value	Coefficient (SE)	*P*-value
Whole body	Sex (female)	−0.0004 (0.0030)	0.9049	–	–
	Body weight	0.0012 (0.0002)	< 0.0001*	–	–
	Injury severity (mild)	−0.0108 (0.0019)	< 0.0001*	−0.0108 (0.0019)	< 0.0001*
	Grip strength	0.0003 (0.0001)	0.0108*	–	–
	Rearing activity	0.0003 (0.0001)	0.0198*	–	–
Lumbar spine	Sex (female)	−0.0059 (0.0050)	0.2551	–	–
	Body weight	0.0017 (0.0005)	0.0022*	–	–
	Injury severity (mild)	−0.0176 (0.0036)	0.0001*	−0.0176 (0.0036)	0.0001*
	Grip strength	0.0005 (0.0002)	0.0161*	–	–
	Rearing activity	0.0003 (0.0002)	0.0964	–	–
Affected femur	Sex (female)	0.0069 (0.0053)	0.2091	–	–
	Body weight	0.0024 (0.0004)	< 0.0001*	0.0024 (0.0004)	< 0.0001*
	Injury severity (mild)	−0.0184 (0.0039)	0.0001*	–	–
	Grip strength	0.0005 (0.0002)	0.0077*	–	–
	Rearing activity	0.0005 (0.0002)	0.0151*	–	–
Affected humerus	Sex (female)	0.0014 (0.0030)	0.6411	–	–
	Body weight	0.0013 (0.0002)	< 0.0001*	0.0013 (0.0002)	< 0.0001*
	Injury severity (mild)	−0.0106 (0.0019)	< 0.0001*	–	–
	Grip strength	0.0003 (0.0001)	0.0066*	–	–
	Rearing activity	0.0003 (0.0001)	0.0050*	–	–

SE: standard error; $R^2 = 0.604$, adjusted $R^2 = 0.586$ in the whole-body BMD analysis; $R^2 = 0.520$, adjusted $R^2 = 0.498$ for lumbar BMD; $R^2 = 0.656$, adjusted $R^2 = 0.640$ for affected femur BMD; $R^2 = 0.653$, adjusted $R^2 = 0.637$ for affected humerus BMD; *: $P < 0.05$ based on linear regression analyses.

main factors affecting bone loss in adults with CP [3,22]. The discovery of the main factors in this translational research became the basis for the development of a mouse model of CP with bone loss.

In the clinical study, ambulatory function was most associated with lumbar and femur bone density, and body weight was an influential factor for determining femur bone density. Non-ambulatory CP participants showed significant bone loss, especially those who were underweight. From previous clinical studies [6,23,24], these two clinical parameters were considered reasonable factors for developing a mouse model of CP with bone loss.

Similar to the clinical study, the factors most affecting skeletal bone loss in the mouse model of CP with neonatal HI brain injury were increased brain injury severity and low body weight [6,23]. Because these results match clinical findings, the translational research hypothesis of a complex interplay of severe brain injury, low body weight, insufficient mechanical loading, and reduced muscle force affecting overall bone health in the mouse model of CP was supported. The osteoporotic condition was clearly characterized by reduced BMD based on the severity of brain insult and the loss of body weight in the mouse model of CP.

Humans and mice have fundamentally different biomechanical loading conditions. Humans primarily engage in upright and bipedal movement, with their weight distributed along the craniocaudal axis, whereas mice primarily engage in quadrupedal movement. Individuals with CP showed obvious non-ambulatory function after severe brain injury, although mice maintained locomotor function [25,26]. In individuals with CP, ambulatory function was strongly associated with femur density and lumbar spine density. In the mouse model of CP, injury severity affected the whole body and lumbar spine density the most, and body weight affected the femur and humerus density the most. In the present study, the factors examined exerted different effects on bone density likely due to the specific types of movement that humans and rodents engage in.

In the transcriptome analysis, *SPP1* expression was significantly downregulated in individuals with CP who had bone loss as compared with normal adults. Thus, bone metabolism in people with CP may be unbalanced due to accumulative lifelong motor impairments. Insufficient mechanical stimuli in individuals with CP can impede bone remodelling. Zhou et al. (2015) [27] suggested that the mechanism of biomineralization by hyper-gravity was attributed to osteopontin expression with enhanced RUNX2 expression. Osteopontin is a non-collagenous protein translated by *SPP1* and contains a collagen-binding motif and arginine-glycine-aspartate (RGD) motif. The CBP exerts biomineralization by binding collagen fibrils with a strong affinity and RGD induces bone resorption [18,28–30]. A synthesized CBP of osteopontin was an effective anti-osteoporotic agent that facilitated osteogenesis in an ovariectomized murine model of postmenopausal bone loss [19]. The CBP has been suggested as an anti-osteoporotic agent based on the underlying mechanism of osteogenesis and anti-adipogenesis [19,31]. Therefore, administering CBP to the mouse model of CP with bone loss was reasonable based on the transcriptome analysis and BMD assessments in human subjects.

Non-ambulatory individuals with CP exhibit abnormal bone change owing to limited mechanical loading showing profound underdevelopment of trabecular microarchitecture [32]. Furthermore, chronic limitations in physical mobility aggravate abnormal symptoms over time, thereby resulting in atypical fractures [33]. Therefore, we expected evidence of skeletal bone loss in patients with CP in this study because these patterns were recently demonstrated [3,34].

However, a remarkable decrease in *SPP1* expression in CP, which results in bone loss as compared with normal adults, was not previously documented in individuals with CP. Therefore, this significant result provided the rationale to implement a novel therapeutic approach by using a synthesized CBP involved in biomineralization in a mouse model of CP with bone loss as a preclinical study.

Before CBP administration, inclusion and exclusion criteria were first used in the mouse model of CP based on 2 main factors: injury severity and body weight. As compared with normal mice, severely HI brain-injured mice with low body weight showed decreased total collagen content relative to corresponding bone areas including trabecular and cortical bone. However, osteoporotic mice in this study showed bone anabolic effects, exhibiting increased total collagen content after long-term CBP treatment as compared with the saline control group. Osteopontin is essential for type-I collagen secretion [35]. Osteopontin mRNA levels were also downregulated in bone-marrow stromal cells from hind-limb disuse in tail-suspended rats as compared with the normal control

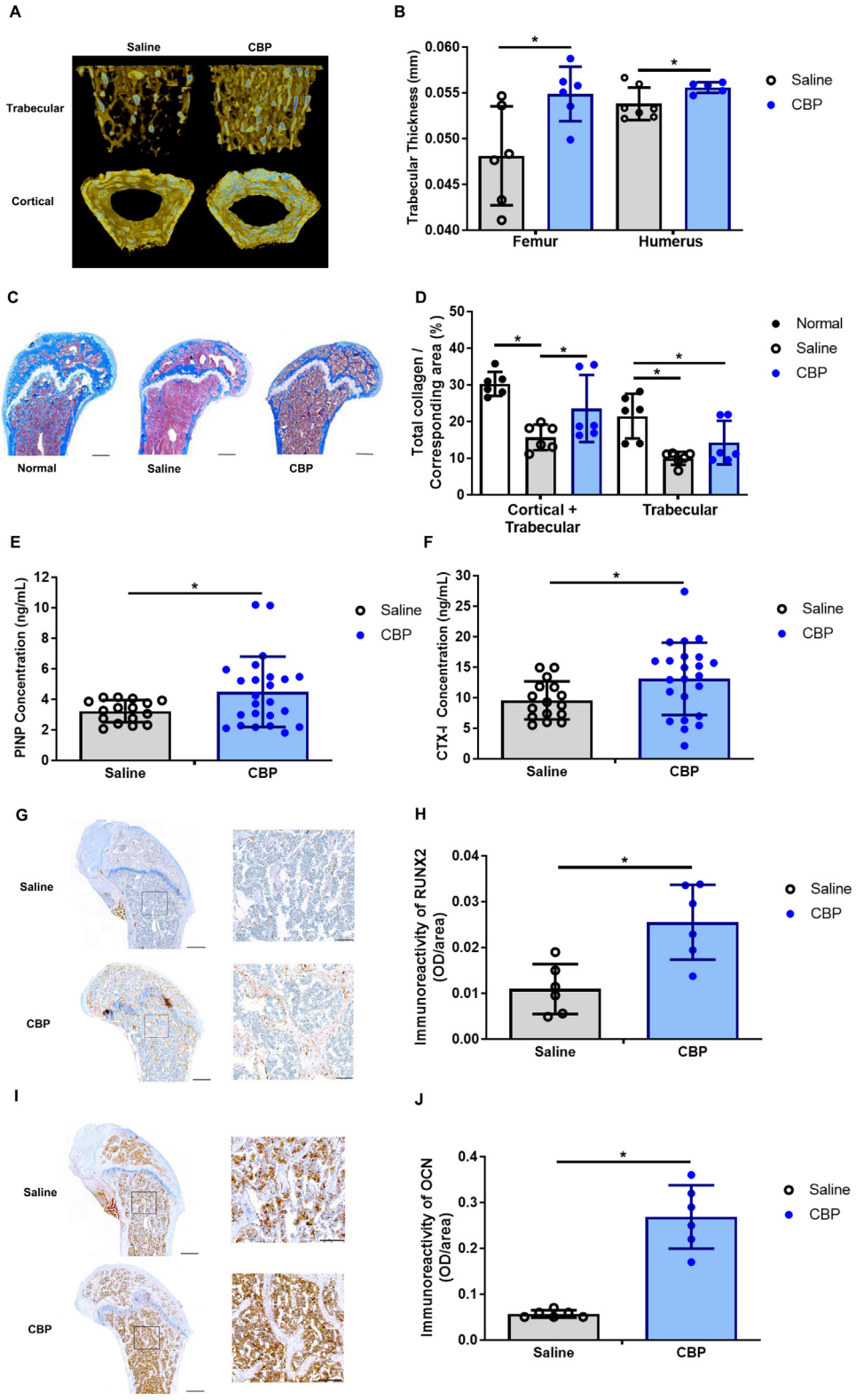

Figure 3. The CBP treatment protected against bone loss in a mouse model of CP. A. A representative 3-D image of femur. B. Trabecular thickness based on micro-CT (μCT) analysis. C. Representative Masson's trichrome for collagen staining. D. Quantification of total collagen to the corresponding area. E and F. Enhanced bone turnover markers PINP and CTX-I were identified in the CBP treatment group compared with the saline control group. G–J. Levels of osteoblast differentiation markers RUNX2 and OCN in the CBP treatment group compared with the saline control group. Data are mean ± SD. *A and B.* Sample size (group in the femur analysis, saline, $n = 6$; CBP, $n = 6$; group in the humerus analysis, saline, $n = 7$; CBP, $n = 5$); *: $P < 0.05$ based on independent *t*-test. *C and D.* Sample size (normal, $n = 6$; saline, $n = 6$; CBP, $n = 6$); scale bar is 500 μm; *: $P < 0.05$ based on *post hoc* comparison after one-way ANOVA. *E and F.* Sample size (saline, $n = 16$; CBP, $n = 24$); PINP, N-terminal propeptide of type I procollagen; CTX-I, C-terminal telopeptide of type I collagen; *: $P < 0.05$ based on independent *t*-test. *G and H.* Scale bar (left: 500 μm; right: 100 μm); RUNX2, runt-related transcription factor 2; sample size (saline, $n = 6$; CBP, $n = 6$); immunoreactivity defined as the ratio of optical density (OD) of immunoreaction sites within the trabecular area. *I and J.* Scale bar (left: 500 μm; right: 200 μm); OCN, osteocalcin; sample size (saline, $n = 6$; CBP, $n = 6$).

[36]. The results indicate that collagen secretion is concomitant with osteopontin expression via mechanical stress in the bone remodelling matrix [37]. On the basis of μCT analysis, the bone morphometrical index of trabecular thickness and 3-D images also demonstrated unhealthy trabecular microstructures in the mouse model of CP. However, CBP treatment significantly improved trabecular thickness in the affected limbs, including femur and humerus.

Bone turnover activity can be altered in individuals with CP, as evidenced by abnormal serum or urine levels of bone formation and bone resorption markers as compared with normal levels. In particular, increased level of the bone resorption marker, CTX-I, is observed [38,39]. Therefore, antiresorptive drugs such as bisphosphonates have been used because of their efficacy in reversing excessive CTX-I level in individuals with CP. However, potentially serious adverse events such as atypical femur fractures are expected when the medications are used for a long time in middle-aged people with CP. In addition, consensus regarding treatment initiation, standardized regimen, and treatment cessation is lacking [40–43].

Unlike bisphosphonates, long-term CBP treatment led to enhanced bone turnover activity in our mouse model of CP with bone loss. Both bone formation and bone resorption was significantly increased in the CBP treatment than saline control group. Although bone turnover markers are difficult to interpret, increased bone turnover activity indicates that CBP treatment was effective in facilitating bone remodelling in the mouse model of CP.

Consistent with the previous study [19], CBP treatment increased the expression of RUNX2, the first transcription factor in the early phase of osteoblast differentiation [44]. OCN expression was also highly enhanced by CBP treatment, regulating osteoblast differentiation and osteoblast maturation in the mouse model of CP with bone loss. Thus, CBP-induced osteogenesis and bone remodelling are linked to biomineralization.

5. Conclusions

From the clinical outcomes of people with CP, in this translational research, a mouse model of CP with bone loss was developed to mimic human disease conditions from bedside to bench. From the transcriptome analyses in human PBMCs, the top-ranked gene *SPP1* was utilized to synthesize the CBP. Anabolic effects of CBP treatment were shown in severely HI brain-injured mice with low body weight. The present study calls for a human trial to provide clinical insights into using CBP medication for treating bone loss in individuals with CP.

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Contribution

YK Shin collected clinical data, conducted experiments to acquire data, analysed data and wrote the manuscript. JH Heo contributed to collecting clinical data. JY Lee and YJ Park contributed to designing experimental condition and provided therapeutic reagents in the animal study. SR Cho designed the translational research, reviewed and finally approved the manuscript.

Disclosure of interest

The authors declare that they have no competing interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.rehab.2020.09.009.

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