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Original Article

Effect of fermented oyster extract on growth promotion in Sprague–Dawley rats

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ABSTRACT

Background: Oysters (*Crassostrea gigas*) are a popular marine product worldwide and have the advantage of nutritional benefits. This study aimed to investigate the effect of fermented oyster extract (FO) on growth promotion, including analysis of body size, bone microarchitecture, hematology and biochemistry *in vivo*.
Methods: The amount of nutrients and gamma aminobutyric acid (GABA) were determined. Sprague–Dawley rats were randomly divided into four groups: the control group, FO 50 group (FO 50 mg/kg), and FO 100 group (FO 100 mg/kg) were administered orally once daily and the recombinant human growth hormone (rhGH) group (200 μg/kg) was intraperitoneally injected once daily for 14 days.
Results: Oral administration of FO 100 significantly increased body length and had no effect on organ damage or hematological profiles. However, administration of rhGH significantly induced hypertrophy of the liver, kidney and spleen along with a marked increase in body length. Tibia length and the growth plate were increased, and bone morphometric parameters were slightly improved by FO and rhGH administration. Serum analysis showed that the levels of GH and insulin like growth factor-1 (IGF-1) were slightly upregulated by FO administration. Nevertheless, the protein expression of hepatic IGF-1 was markedly increased by FO 100 and rhGH administration.
Conclusions: FO have high content of GABA, and induced positive effects on body length, tibial length, growth-plate length and hepatic IGF-1 synthesis in SD rats with no toxicity or alterations of hematological profile. Therefore, these results suggest that GABA-enriched FO could be considered a potential alternative treatment for growth stimulation.

1. Introduction

Body growth is controlled by environmental factors and individual conditions, including nutritional, hormonal and genetic status, which interact to form a complex process across all organs.[1,2] Some therapies are available to improve growth, including recombinant human growth hormone (rhGH), insulin like growth factor-1 (IGF-1), aromatase inhibitors (anastrozole and letrozole) and bone lengthening surgery.[2] rhGH therapy has been widely used for clinical purposes for more than fifty years.[2,3] Nevertheless, there are reported adverse effects of rhGH administration in children and adolescents, including edema, insulin resistance, progression of scoliosis, prepubertal gynecomastia, benign intracranial hypertension, etc.[3] In this respect, aromatase inhibitors can be considered

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as alternative oral treatment strategies for short stature.[4] However, several studies have found that aromatase inhibitors have toxicity, although they improved the predicted final height in children.[5,6] Therefore, there is a growing need to develop new and effective oral replacements that have low side effects and are safe for the treatment of growth failure.

Oysters are a popular marine product worldwide and have the advantage of nutritional benefits.[7,8] Oysters and its derivative have long been used as a traditional medicine in Southeast Asia for the treatment of acid indigestion, fatigue, hemorrhage and gastrointestinal troubles.[9] Numerous studies have suggested that contents derived from oysters have bioactivity on bone health including osteogenic activity[10] and improving bone growth mediators.[11] We recently demonstrated that fermented oyster (*Crassostrea gigas*) extract (FO) prevents osteoclast differentiation, inhibits ovariectomy (OVX)-induced bone loss and decreasing of bone morphometric parameters,[12,13] and promotes bone formation.[7] Although previous studies of the effects of FO on stimulation of osteogenesis and suppression of osteoclastogenesis, no studies have established the effect of FO as a replacement for growth failure. Therefore, the aim of this study was to evaluate the effect of FO on growth promotion, including an analysis of growth parameters, hematology and biochemistry in Sprague–Dawley rats.

2. Methods

2.1. Preparation of FO

FO used in this study was obtained from Marine Bioprocess Co. Ltd. (Busan, Republic of Korea). FO was prepared by fermentation with *Lactobacillus brevis* BJ20 as previously described with slight modifications,[13] namely, glutamic acid and dextrin were used as a substitute for monosodium glutamate and an excipient, respectively. Prior to use in the experiments, the FO was diluted with distilled water to adjust the final treatment concentrations.

2.2. Chemical analyses of FO

The amounts of nutrients, such as carbohydrates, crude proteins and sugars, were determined by the methods of the Food Code of Korea.[14] The content of gamma aminobutyric acid (GABA) was determined using a high-performance liquid chromatography (HPLC) system (Dionex U3000; Thermo Sci., Sunnyvale, CA, USA) equipped with a UV detector. Dionex Bonded Silica column (C_{18} 5 μm 120 Å 4.6 × 250 mm) fitted with 4.0 × 3.0 mm i.d. guard column, both from Phenomenex (Torrance, CA), were used. Solvent A was 50 mM sodium acetate (pH 6.5), and solvent B consisted of 45% (v/v) acetonitrile, 45% (v/v) methanol and 10% (v/v) distilled water. The linear gradient was conducted for 30 min at 338 nm, and the injection volume was 20 μL. The solvent flow rate was 1.0 mL/min. All other chemicals used were of analytical grade, and were purchased from the Sigma–Aldrich Chemical Co.

2.3. Animal and experimental procedures

This study was conducted in accordance with the animal experimentation guidelines of Dong-eui University, with approval of the Institutional Animal Care and Use Committee (No. R2019-002) for the use of animals in research. We purchased 79 Sprague–Dawley (SD) rats (female, postnatal day 21) from Samtako Bio Korea (Osan, Republic of Korea). After acclimatization for 7 days, all rats were randomly divided into four groups: the control group (n = 19, 100 μL of distilled water), FO 50 group (n = 20, 100 μL of 50 mg/kg/day), FO 100 group (n = 20, 100 μL of 100 mg/kg/day), and rhGH group (n = 20, 200 μg/kg/day). The control and FO groups were administered orally once per day in the morning for 14 days. The feeding dose of FO was determined based on the effective dosage of previously report.[12] The rhGH group, as a positive control,[15] was subcutaneously injected once daily for 14 days. rhGH was obtained from Dong-A ST Co, Ltd. (Growtropi$^{n®}$-II, Seoul, Republic of Korea). The body weight was measured weekly. All rats were sacrificed at day 14 after treatment, and the body length was defined separately as the length from the nose to the tail (N–T) and the length from the nose to the anus (N–A).

2.4. Collection of blood and tissue

Whole Blood was collected directly from the heart, placed in heparinized tube, allowed to clot for 30 min at room temperature. Thereafter the blood centrifuged to obtain the serum at 3000 rpm for 10 min at 4 °C, which was kept at −80 °C for subsequent analysis. After perfusion, organs were immediately surgically excised, including the thymus, heart, lung liver, kidney, spleen, uterus and ovary, weighed, and stored at −80 °C. The long bones (femurs and tibias) were dissected out and fixed in 4% paraformaldehyde.

2.5. Micro-CT and histomorphometric analysis

Bone morphometric parameters of rat tibias were evaluated using high-resolution micro-computed tomography (μCT, Skyscan 1272; Kontich, Belgium) with a source voltage of 80 kV, current of 125 μA and resolution of 12 μm. Bone volume per total volume (BV/TV), bone mineral density (BMD), trabecular separation (Tb. Sp.), and trabecular number (Tb. N.) were measured using CTAn software (Bruker; Kontich, Belgium) as reported previously.[16,17] For histomorphometric analysis, fixed tibias were decalcified in 12% ethylenediaminetetraacetic acid and embedded in paraffin. Longitudinal tissue sections were prepared using a microtome (Leica Biosystems, Nussloch, Germany) with 5 μm and stained with hematoxylin and eosin (H&E). For growth-plate analysis, mid regions of the tibia were selected, and the length of the upper and lower growth plate was measured using iSolution software (Daejeon, Korea) at 10× magnification. At least 10 regions of the growth plate were measured for each section.[18]

2.6. Hematology

Red blood cells (RBC), white blood cells (WBC), hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC) and platelet were analyzed with a Sysmex XN-9000 analyzer (Sysmex Corporation, Kobe City, Hyogo Prefecture, Japan).

2.7. Serum biochemistry

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine and calcium were measured using the Cobas 8000 C702 chemistry analyzer (Roche, Mannheim, Germany).

2.8. Serum GH, IGF-1 and IGFBP-3 levels

Serum GH (catalog No. EMIGFBP3), insulin like growth factor-1 (IGF-1, catalog No. OKBB00165) and insulin like growth factor binding protein-3 (IGFBP-3, catalog No. OKBB00172) levels were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The GH ELISA kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). IGF-1 and IGFBP-3 kits were purchased from AVIVA Systems Biology (San Diego, CA, USA).

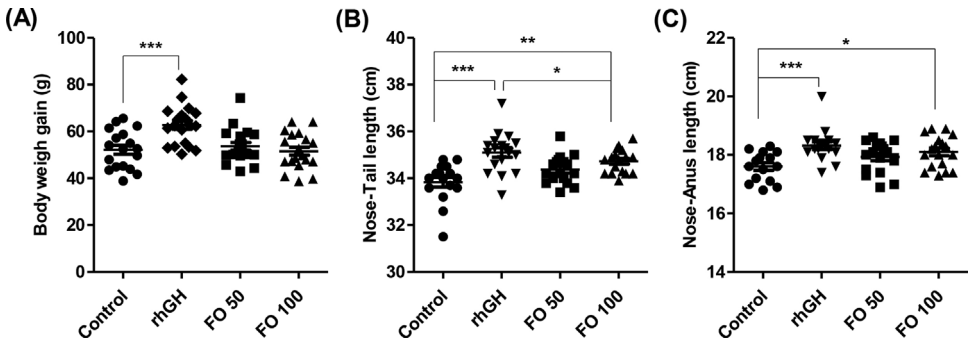

Fig. 1. Effects of FO on body weight and body length in SD rats. (A) The body weight gain of each group after 2 weeks of treatment. (B and C) The length from the nose to tail and the nose to anus of each group after 2 weeks of treatment. Scatter plot graphs show the means ± standard deviation (SD, $n = 19$). Statistical analyses were conducted using analysis of ANOVA-Tukey's *post hoc* test between groups. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Table 1
Changes in the Weight of Organs After 2 Weeks of Treatment

Group	Weight of organs (g)						
	Thymus	Heart	Lung	Liver	Kidney	Spleen	Uterus Ovary
Control	0.46 ± 0.09	0.60 ± 0.05	0.88 ± 0.09	6.09 ± 0.50	1.40 ± 0.10	0.45 ± 0.08	0.39 ± 0.07
rhGH	0.47 ± 0.07	0.62 ± 0.06	0.93 ± 0.10	7.88 ± 1.61*	1.63 ± 0.29*	0.51 ± 0.06*	0.41 ± 0.11
FO 50	0.48 ± 0.06	0.61 ± 0.03	0.88 ± 0.08	6.60 ± 0.95	1.42 ± 0.11	0.44 ± 0.02	0.48 ± 0.14
FO 100	0.38 ± 0.05	0.57 ± 0.05	0.85 ± 0.08	6.55 ± 1.02	1.46 ± 0.12	0.43 ± 0.08	0.38 ± 0.06

Mice were sacrificed at day 14 after treatment. Thymus, heart, lung, liver, kidney, spleen, uterus, and ovary were immediately surgically excised, and the weights were then measured. The data are expressed as the means ± standard deviation (SD, $n = 19$). The statistical analyses were conducted using analysis of ANOVA-Tukey's *post hoc* test between groups.

* $p < 0.05$ compared to control.

2.9. Western blot analysis

Proteins from rat liver were extracted using the Bradford Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Total protein (50 μg) was separated by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After electrophoresis, separated proteins were transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Triton X-100 (TBST) for 1 h at room temperature. The membranes were probed with mouse monoclonal anti-IGF-1 antibody (catalog No. sc-74116, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. After washing, the membranes were incubated with goat-anti-mouse IgG-HRP antibody (catalog No. sc-2005, Santa Cruz Biotechnology) for 1 h at room temperature as previously described.[19] Protein expression of IGF-1 was detected by an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Little Chalfont, UK) and visualized by a Fusion FX Image system (Vilber Lourmat, Torcy, France). All other chemicals used were of analytical grade, and were purchased from the Sigma–Aldrich Chemical Co.

2.10. Statistical analysis

The data are expressed as the means ± standard deviation (SD) and were analyzed using GraphPad Prism software (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). Differences between groups were assessed using analysis of variance followed by one-way ANOVA-Tukey's *post hoc* test, and $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Proximate composition of FO

The proximate analysis showed that FO is composed of 46 g/100 g carbohydrate, 36 g/100 g crude protein, 6.3 g/100 g sugars and 114 mg/g GABA (Supplementary Table S1 and Figure S1).

3.2. Effect of FO on body weight and body length in SD rats

As shown in Fig. 1A, the body weight gains of the FO 50 (39.73 ± 3.46 g) and FO 100 (38.67 ± 4.90 g) groups were similar to those of the control group (37.91 ± 4.32 g). However, the body weight gain was significantly increased in the rhGH group (46.43 ± 5.53 g, $p < 0.001$) compared to that in the control group. In the FO 100 group, the N–T length and the N–A length were significantly increased to 34.73 ± 0.51 cm ($p < 0.01$) and 18.10 ± 0.54 cm ($p < 0.05$), respectively, compared to the control group (Fig. 1B and C). Furthermore, rhGH-treated rats showed marked increases in the N–T length (35.10 ± 0.85 cm, $p < 0.001$) and N–A length (18.32 ± 0.55 cm, $p < 0.001$) compared to control rats.

3.3. Effect of FO on organ weight in SD rats

The weights of selected organs were not significantly different between FO-treated groups and the control group (Table 1). Hypertrophies of the liver (1.30-fold of control, $p < 0.05$), kidney (1.22-fold of control, $p < 0.05$) and spleen (1.20-fold of control, $p < 0.05$) were significantly induced in rhGH-treated rats compared to control rats.

3.4. Effect of FO on hematological and biochemical profiles in SD rats

An analysis of RBC, WBC, hematocrit, hemoglobin, MCV, MCH, MCHC and platelets showed no differences among the groups (Table 2). Furthermore, no biochemical abnormalities, including the levels of serum ALT, AST, BUN, creatinine and calcium, were observed among the groups.

Table 2
Changes in the Hematological and Biochemical Profiles After 2 Weeks of Treatment

Parameter (units)	Group			
	Normal	rhGH	FO 50	FO 100
RBC (10^6/μL)	6.67 ± 0.39	6.60 ± 0.19	6.79 ± 0.15	6.92 ± 0.30
WBC (10^3/μL)	3.18 ± 0.45	2.85 ± 0.42	2.66 ± 0.96	3.75 ± 0.96
Hematocrit (%)	48.81 ± 2.86	48.69 ± 1.26	49.46 ± 1.41	50.08 ± 2.29
Hemoglobin (g/dL)	14.24 ± 0.66	14.04 ± 0.28	14.31 ± 0.49	14.21 ± 0.59
MCV (fL)	73.16 ± 1.52	73.70 ± 1.93	72.90 ± 1.40	72.56 ± 1.40
MCH (pg)	21.34 ± 0.48	21.36 ± 0.59	21.06 ± 0.63	20.94 ± 0.34
MCHC (g/dL)	29.16 ± 0.40	28.88 ± 0.44	28.89 ± 0.57	28.99 ± 0.31
Platelet (10^3/μL)	1101.38 ± 257.49	1219.63 ± 130.72	1222.88 ± 218.25	1360.38 ± 181.24
ALT (U/L)	40.70 ± 7.09	39.50 ± 6.87	35.20 ± 6.89	37.90 ± 6.81
AST (U/L)	100.20 ± 11.42	92.40 ± 14.18	105.10 ± 11.42	101.70 ± 10.14
BUN (mg/dL)	14.36 ± 2.93	12.52 ± 1.38	13.51 ± 1.62	12.76 ± 1.26
Creatinine (mg/dL)	0.29 ± 0.05	0.28 ± 0.02	0.29 ± 0.03	0.29 ± 0.03
Calcium (mg/dL)	11.45 ± 0.59	11.44 ± 0.31	11.14 ± 0.25	11.22 ± 0.27

At day 14 after treatment, whole blood and serum were analyzed for hematological and biochemical evaluation. The data are expressed as the means ± standard deviation (SD, $n = 19$). The statistical analyses were conducted using analysis of ANOVA-Tukey's *post hoc* test between groups. All data showed that there is no statistically significant difference between groups. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen, MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration; RBC, red blood cells; WBC, white blood cells.

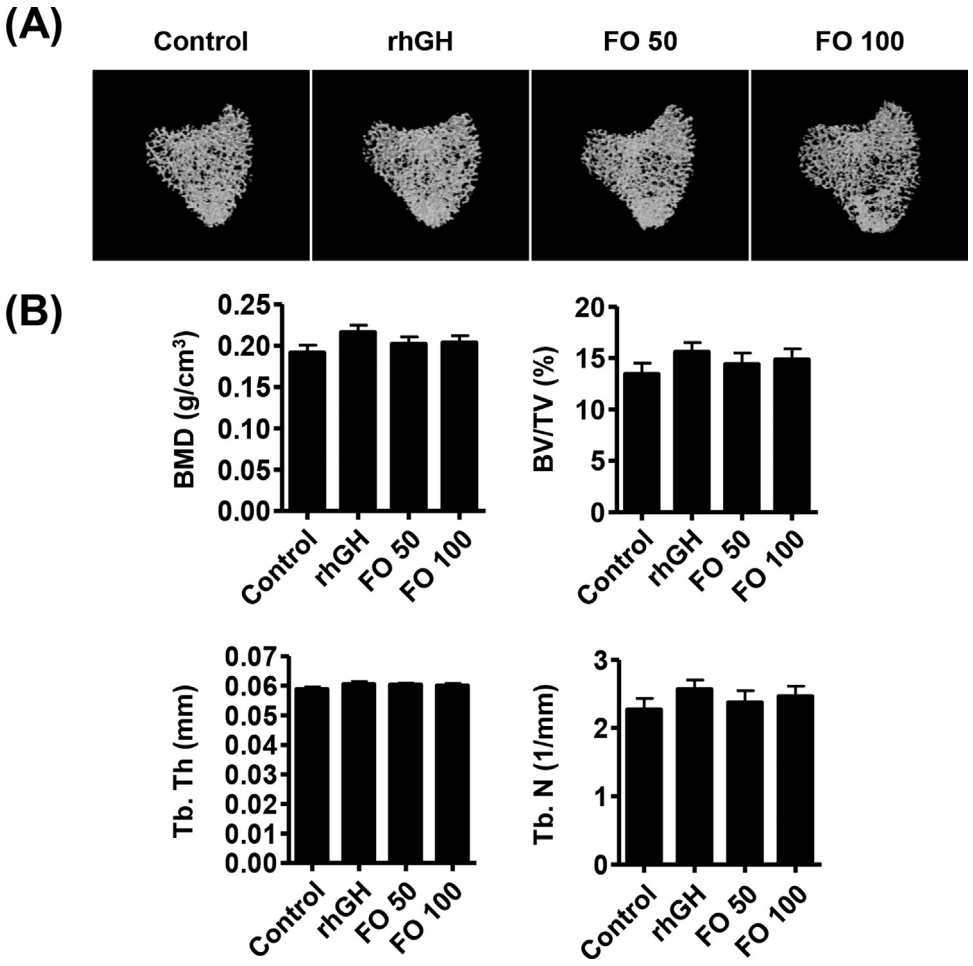

Fig. 2. Effects of FO on tibial trabecular bone microarchitecture in SD rats. Bone morphometric parameters were analyzed using high-resolution micro-computed tomography (μCT, Skyscan 1272; Kontich, Belgium). (A) Micro-CT images of tibial trabecular bone. (B) Analysis of bone morphometric parameters, such as bone volume per total volume (BV/TV), bone mineral density (BMD), trabecular thickness (Tb. Th), and trabecular number (Tb. N). The data are expressed as the means ± standard deviation (SD, $n = 19$).

3.5. Effect of FO on tibial trabecular bone microarchitecture in SD rats

The results of μCT analysis showed that BMD, BV/TV and Tb.N were slightly increased in the rhGH and FO groups, but there was no statistically significant difference compared with the control group (Fig. 2A and B). In addition, there was no predominated concentration-dependent effect between FO 50 and FO 100 administration.

3.6. Effect of FO on proximal tibial growth plate in SD rats

No statistically significant difference was observed in the rhGH group compared with the control group, although proximal tibial

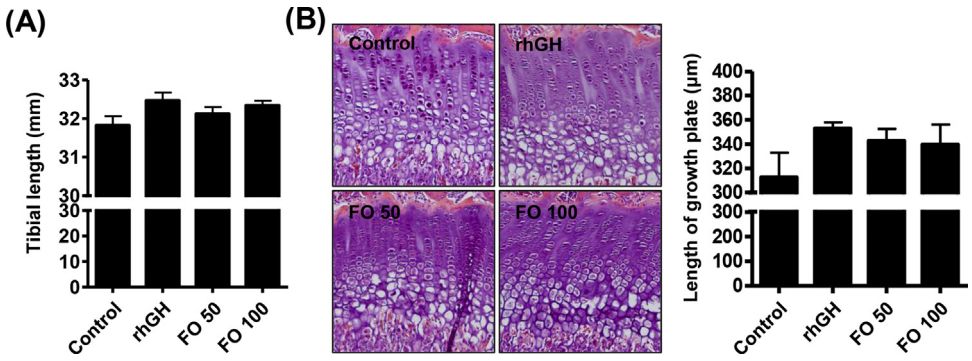

Fig. 3. Effects of FO on the proximal tibial growth plate in SD rats. (A) Tibial length. (B, left) Representative photographs of H&E-stained chondrocytes of the proximal tibial growth plate in SD rats. (B, right) Length of the proximal tibial growth plate. The data are expressed as the means ± standard deviation (SD, $n = 19$).

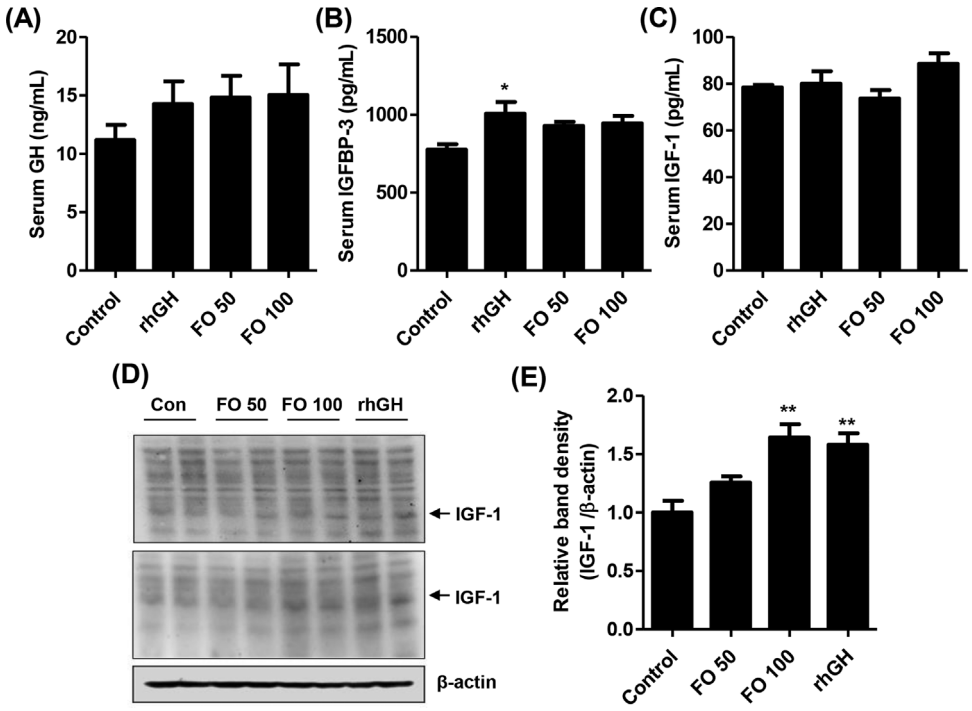

Fig. 4. Effects of FO on growth parameters in SD rats. (A–C) Serum GH, IGFBP-3 and IGF-1 levels. The serum growth parameters were measured using ELISA kits. The data are expressed as the means ± standard deviation (SD, $n = 19$). The statistical analyses were conducted using analysis of ANOVA-Tukey's *post hoc* test. $^{*}p < 0.05$ compared to control. (D) The protein expression of hepatic IGF-1. β-actin served as the loading control. (E) Bar graphs indicate the relative band density of the IGF-1 in western blot analysis. The statistical analyses were conducted using analysis of ANOVA-Tukey's *post hoc* test. $^{**}p < 0.01$ compared to control.

length was slightly increased. Proximal tibial length was partially lengthened in the FO 100 group (32.34 ± 0.43 mm, Fig. 3A). The effect of FO on the increased proximal tibial length was dose dependent. The length of the proximal tibial growth plate gradually increased with FO 50 (342.73 ± 14.02 μm) and FO 100 (and 339.55 ± 28.63 μm) administration, and these increasing levels were similar to those in the rhGH group (352.97 ± 6.87 μm) (Fig. 3B).

3.7. *Effect of FO on growth parameters in SD rats*

The ELISA results for serum GH levels indicated that, although FO 50, FO 100 and rhGH groups showed a growing trend after 2 weeks of administration, there were no significant differences among the groups (Fig. 4A). In addition, serum IGFBP-3 levels slightly increased with FO administration, but those levels were not significantly different compared with those in the control group (Fig. 4B). However, rhGH treatment gradually upregulated serum IGFBP-3 levels (1.30-fold that of control, $p < 0.05$) in SD rats. Serum IGF-1 levels were highest in the FO 100 group (88.56 ± 17.73 pg/mL, Fig. 4C). However, there was no significant difference in serum IGF-1 levels between control and rhGH-treated rats (Fig. 4C). The protein expressions of hepatic IGF-1 were markedly upregulated by FO 100 (1.64-folds of control, $p < 0.01$) and rhGH administration (1.58 folds of control, $p < 0.01$, Fig. 4C).

4. Discussion

In the present study, we investigated the effect of FO on growth promotion, including analyses of body size, bone microarchitecture, hematology and biochemistry in SD rats. Our results show that oral administration of FO 100 significantly increased body lengths, including N–T and N–A. These results are meaningful in that those body lengths are the first criteria used to determine the growth in height. Additionally, our data indicate that BMD, BV/TV and Tb.N were slightly increased in the FO groups compared with

the control group. Furthermore, in the FO 100 group, the lengths of the proximal tibia and proximal tibial growth plate gradually increased. Meanwhile, administration of rhGH, as a positive control, also improved the N–A, N–T, proximal tibia and proximal tibial growth plate lengths in SD rats. However, the administration of rhGH increased body length and proximal tibial growth-plate length but also induced body weight increases and organ hypertrophy. These are consistent with previous human and animal studies.[3,20–24] Based on the present study, administration of FO induced increased body length and proximal tibial growth-plate length without organ damage in SD rats. Additionally, our results show that the levels of serum GH and IGFBP-3 are slightly upregulated by FO administration. Nevertheless, the protein expression of hepatic IGF-1 was markedly increased by FO 100 and rhGH administration. These results suggest that, although administration of FO did not affect circulating serum IGF-1 levels, IGF-1 synthesis occurred in the liver.

GABA has been directly implicated in the regulation of muscle tone[25] and stimulated osteoblastogenesis by upregulating bone formation genes in ovariectomized rats.[26] Recently, one study showed that GABA-enriched fermented sea tangle (FST) significantly increased human growth hormone and IGF-1 levels[27] and muscle-related growth factors.[28] Our previous report found that GABA is efficiently produced through biotransformation from glutamic acid during fermentation with *L. brevis* BJ20.[29] From these results, it is estimated that FO, including GABA, can enhance growth parameters, such as hepatic IGF-1 synthesis as well as body lengths, in SD rats.

In current study, our findings shown that that FO have high content of GABA, and increases body length and hepatic IGF-1 synthesis in SD rats without toxicity. Nevertheless, the limitations of this study are no statistical significance although bone morphometric parameters and serum growth factors were slightly upregulated by FO administration. Therefore, further studies are needed to assess the efficacy of FO at high doses and in long-term animal models.

Author contributions

Conceptualization: B-JL, GYK, EKP and YHC. Methodology: JSN, J-HP and B-JL. Validation: J-HP and B-JL. Formal Analysis: MW, Y-SK and Y-CC. Investigation: HL, HH, SYJ, MYK, SYK, G-YK and EKP. Writing - Original Draft: HL and YHC. Writing - Review & Editing: YHC. Visualization: JSN. Supervision: YHC. Funding Acquisition: GYK and Y-JJ.

Conflict of interest

The authors declare no conflicts of interest.

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Ethical statement

This study was conducted in accordance with the animal experimentation guidelines of Dong-eui University, with approval of the Institutional Animal Care and Use Committee (No. R2019-002).

Data availability

Data and materials will be available from the authors upon request.

Supplementary materials

Supplementary Fig. S1. HPLC chromatogram for γ-amino butyric acid of fermented oyster extract and Supplementary Table S1 Proximate composition of FO can be found in the online version at doi:10.1016/j.imr.2020.100412.

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