

Upregulation of genes related to bone formation by γ -amino butyric acid and γ -oryzanol in germinated brown rice is via the activation of GABA_B-receptors and reduction of serum IL-6 in rats

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Background: Osteoporosis and other bone degenerative diseases are among the most challenging non-communicable diseases to treat. Previous works relate bone loss due to osteoporosis with oxidative stress generated by free radicals and inflammatory cytokines. Alternative therapy to hormone replacement has been an area of interest to researchers for almost three decades due to hormone therapy-associated side effects.

Methods: In this study, we investigated the effects of gamma-amino butyric acid (GABA), gamma-oryzanol (ORZ), acylated sterol glucosides (ASG), and phenolic extracts from germinated brown rice (GBR) on the expression of genes related to bone metabolism, such as *bone morphogenic protein-2 (BMP-2)*, *secreted protein acidic and rich in cysteine (SPARC)*, *runt-related transcription factor 2 (RUNX-2)*, *osteoblast-specific transcription factor osterix (Osx)*, *periostin*, *osteoblast specific factor (Postn)*, *collagen 1&2 (Col1&2)*, *calcitonin receptor gene (CGRP)*; body weight measurement and also serum interleukin-6 (IL-6) and osteocalcin, in serum and bone. Rats were treated with GBR, ORZ, GABA, and ASG at (100 and 200 mg/kg); estrogen (0.2 mg/kg), or remifemin (10 and 20 mg/kg), compared to ovariectomized non-treated group as well as non-ovariectomized non-treated (sham) group. Enzyme-linked immunosorbent assay was used to measure the IL-6 and osteocalcin levels at week 2, 4, and 8, while the gene expression in the bone tissue was determined using the Genetic Analysis System (Beckman Coulter Inc., Brea, CA, USA).

Results: The results indicate that groups treated with GABA (100 and 200 mg/kg) showed significant upregulation of *SPARC*, *calcitonin receptor*, and *BMP-2* genes ($P < 0.05$), while the ORZ-treated group (100 and 200 mg/kg) revealed significant ($P < 0.05$) upregulation of *Osx*, *Postn*, *RUNX-2*, and *Col1&2*. Similarly, IL-6 concentration decreased, while osteocalcin levels increased significantly ($P < 0.05$) in the treated groups as compared to ovariectomized non-treated groups.

Conclusion: GABA and ORZ from GBR stimulates osteoblastogenesis by upregulation of bone formation genes, possibly via the activation of GABA_B receptors and by inhibiting the activity of inflammatory cytokines and reactive oxygen species. Therefore, it could be used effectively in the management of osteoporosis.

Keywords: gene expression, GBR-bioactive compounds, osteocalcin, ovariectomized rats

Introduction

Diet plays an important role in modifying gene expression in both healthy and diseased conditions. The changes and modifications in gene expression in bone tissue explain a developmental sequence that has three principal periods: the proliferation stage,

extracellular matrix maturation, and mineralization period.^{1,2} Bone metabolism is a complex mechanism controlled by a number of factors including genetic, environmental, and lifestyle.³ Genetic factors play a significant role in the development of skeletal malformations which have been estimated to account for 70%–80% of the variance in bone density (BMD), and also 25%–35% risk for bone fractures.^{4–6} Cells in bone tissues are divided into the osteoblast, which are mainly involved in bone formation and mineralization;⁷ they are derived from pluripotent mesenchymal stem cells which differentiate into chondrocytes, adipocytes, myoblasts, fibroblasts, osteoclasts, and osteocytes.^{8,9} The osteocytes play a role in responding to mechanical stimuli, which initiate a modeling or remodeling response, while the osteoclasts are bone resorbing cells from hematopoietic precursors of the monocyte/macrophage lineage and are associated with osteoclastogenesis.^{7,10,11} Bone morphogenic protein-2 (BMP-2) is a potent osteo-inductive factor which induces the osteogenic differentiation of mesenchymal cells,^{12,13} secreted protein acidic and rich in cysteine (SPARC) is a secreted calcium-binding glycoprotein that regulates mineralization of bone tissues in mammals,^{14,15} and runt-related transcription factor 2 (RUNX-2) plays an important role in osteogenesis.^{16,17} Osterix (OSX) has been described as a transcriptional factor for osteoblast differentiation.^{18,19} Osteocalcin is a major non-collagenous protein of the bone matrix—osteoblasts synthesize osteocalcin and after production, part of the osteocalcin is incorporated into the bone matrix while the other part goes into the circulatory system. Circulating osteocalcin is regarded as a specific marker for bone formation due to its association with the changes in the rate of bone turnover.²⁰ Interleukin-6 (IL-6) is an inflammatory marker that is strongly associated with an increase in estrogen (EST) deficiency, especially in osteoporosis.²¹ Germinated brown rice, a brown rice subjected to sporulation, has been reported to increase the concentration of its bioactives. The most important is the natural tranquilizer, gamma-amino butyric acid (GABA), which is an amino acid and a neurotransmitter that fosters communication between nerve cells. It improves relaxation and sleep and also plays an active role in seizure prevention and alleviation of chronic pain.²² GABA is known as the major inhibitory neurotransmitter found in the central nervous system. Its major role is the regulation of neuron excitability in the nervous system and it has been directly implicated in the regulation of muscle tone.²³ The concentration of GABA in brown rice increases dramatically to about 10-fold after germination.^{24–28} Oryzanol (ORZ), which has been described as a powerful antioxidant, has also been shown to increase

during the process.²⁵ In the present work, we studied the expression of seven genes related to bone metabolism in ovariectomized (OVX) rats treated with germinated brown rice (GBR)-phenolics, ORZ, GABA, acylated steryl glucosides (ASG), remifemin (REM), or EST to determine the role of these compounds in stimulating bone formation.

Materials and methods

Brown rice, drugs, and chemicals

Brown rice (BR) is from the Malaysian rice variety (MR220) which was supplied by PadiBeras Nasional (BERNAS) factory (Sri Tiram Jaya, Malaysia). The germination procedures were carried out as explained in our previous publication.²⁹ GABA was extracted and quantified using a high performance liquid chromatography diode array detector (Agilent Technologies, Santa Clara, CA, USA) and the same principles as described by Rozan et al,³⁰ while ORZ was analyzed applying the method reported by Azlan et al.³¹ ASG was extracted and analyzed as reported by Usuki et al.³² *Cimicifuga racemosa* (Remifemin® 20 mg/tab) was purchased from Schaper and Brummer (Salzgitter, Germany). The conjugated EST (Premarin® 0.625 mg/tab) was procured from Wyeth Ireland Newbridge, Co (Kildare, Ireland) and the Rat-MID Osteocalcin enzyme immunoassay (EIA) (Ref no AC-12F1) was from Immunodiagnostic Systems (Baldon Colliery, UK). The Rat IL-6 enzyme-linked immunosorbent assay (ELISA) kit (Ref no ER3IL6), was from Thermo Scientific (Rockford, IL, USA).

Experimental animals, grouping, and dosing

Sprague Dawley rats weighing approximately 250–260 g were used in this study. They were divided into 13 groups of six rats each (78 rats). The rats were acclimatized for 2 weeks before commencement of the experiment. The study was carried out according to the guidelines for the use of animals as approved by the Animal Care and Use Committee Faculty of Medicine with approval number UPM/FPSK/PADS/UUH/F01, University Putra Malaysia. The rats in group 1 were sham operated by exposing the ovaries and returning them back to their anatomical position. Bilateral ovariectomy was performed on the rats in groups 2–13 under general anesthesia. Treatments were given orally, starting from 2 weeks after the surgery, and were given once daily for a period of 8 weeks. Group 2 rats were OVX without treatment; groups 3, 4, and 5 were treated with 0.2 mg/kg EST, 10 and 20 mg/kg REM, respectively; groups 6 and 7 rats were treated with GBR-phenolics at the dose rates of 100 and 200 mg/kg, respectively; groups 8 and 9 were treated with ASG at 100 and 200 mg/kg, respectively; groups 10 and 11 were treated with 100 and 200 mg/

kg of GABA, respectively; while groups 12 and 13 were each treated with ORZ at 100 and 200 mg/kg, respectively.

Body weight measurements

Rats were weighed using a weighing scale before the surgery, 2 weeks after the surgery just before the commencement of the treatments, and at weeks 2, 4, and 8 after the commencement of the treatments.

IL-6 and osteocalcin ELISA assays

Serum IL-6

The Rat IL-6 ELISA kit was used for the assay as per the manufacturer instructions. Briefly, 100 μ L of the standard or serum samples was added to each well of the 96-well plate, incubated for 2 hours at 20°C–25°C, and washed five times with water. Biotinylated antibody (100 μ L) was added to each well, the plate was covered, and incubated at room temperature for 1 hour. Streptavidin-horseradish peroxidase solution (100 μ L) was then added to each well after washing and incubated at room temperature for 30 minutes. Plates were washed and 100 μ L of 3,3',5,5'-tetramethylbenzidine substrate was added to each well before developing in the dark for 30 minutes; 50 μ L of the stop solution was then added to each well, and the absorbance was read at 450 nm using a spectrophotometer, Thermo LabSystem ELISA reader OPSYS MR (Thermo life science, Basingstoke, UK).

Serum osteocalcin level

Rat-MID osteocalcin EIA, was used for osteocalcin quantification, following the manufacturer instructions. Briefly, 100 μ L of biotinylated osteocalcin was added to each well of a streptavidin pre-coated 96-well plate, covered using sealing tape, incubated for 30 minutes at 20°C on a microtiter mixing apparatus (300 rpm), and washed five times with washing solution. Twenty milliliters of the standard, control, or the unknown serum sample were pipetted into the appropriate wells followed by the addition of the primary antibody (150 μ L), which was earlier prepared by mixing the primary antibody and primary incubation buffer in a ratio of 1:100. The strips were covered and incubated for 1 hour at room temperature on a mixing apparatus; the plates were then washed before the addition of the secondary antibody (100 μ L). These were covered and incubated for another hour at room temperature. The plates were washed and a substrate solution (100 μ L) was added to each well and incubated for 15 minutes at room temperature in the dark on the plate mixing apparatus. The reaction was then stopped by adding 100 μ L of the stopping solution to each well and the absorbance was measured using

a spectrophotometer at 450 nm using 650 nm as the reference. All solutions were equilibrated to room temperature.

RNA isolation

RNA (ribonucleic acid) was isolated from the bone tissue immediately after sacrifice using the HiYield Total RNA Mini Kit[®] (Real Biotech, Taipei, Taiwan) according to the manufacturer's instructions. Briefly, 25 mg of bone tissue from the femur was cracked using a bone cracker and then immediately transferred into a 1 mL Eppendorf tube containing 400 μ L of lysis buffer on ice. β -mercaptoethanol (4 μ L) was added, and a micropestle was used to grind the tissue for about 3 minutes, and then incubated at room temperature for 5 minutes. The sample was transferred to a lysate filter column placed on a 2 mL collection tube and centrifuged at 1000 rpm/4°C for 1 minute. Seventy percent absolute ethanol (400 μ L) was added to the filtrate and the mixture was shaken vigorously. The ethanol added mixture was then transferred to an RNA binding column placed in a 2 mL collection tube and centrifuged at 12,000 rpm/4°C for 2 minutes, and the flow-through 25 μ L of DNase was then added to the center of the column and left to stand for 20 minutes.

RNA yield, purity, and integrity

RNA yield is reported based on the absorbance at 260 nm using a Nanodrop spectrophotometer which was further confirmed using the Agilent RNA 6000 Nano Lab-Chip Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies). The quality of the isolated RNA was determined using the Agilent 2100 Bioanalyzer; an absorbance ratio at 260 nm and 280 nm, along with the ratio of the absorbance at 260 nm and 230 nm were used in detecting the quality. Quantification of the RNA quality is reported in an RNA Integrity Number (RIN), which is the total RNA produced in the analysis. A RIN of more than or equal to 7 indicates that the RNA is suitable for gene expression study.^{8,9}

Primer design

Primers stocks were diluted in nuclease-free water to a concentration of 500 nM for the reverse primer, and 200 nM for the forward primer. The size ranged from 145–224 nucleotides, with a 6-nucleotide minimum separation size between polymerase chain reaction (PCR) products. In addition to the seven genes of interest, each panel contained an internal control gene and normalization genes. The reverse primers consisting of 20 nucleotides complementary to the target were tagged to a 19-nucleotide universal reverse sequence, while the forward primers consisted also of 20 nucleotides corresponding to the

Table 1 Serum IL-6 concentration (pg/mL) in OVX rats treated with GBR-phenolic compounds, ASG, GABA, ORZ, EST, or REM over a period of 8 weeks

Treatment groups (mg/kg)	IL-6 concentration (pg/mL)		
	Week 2	Week 4	Week 8
ASG, 100	112.81 ± 2.60 ^{de}	156.67 ± 5.15 ^b	141.51 ± 17.37 ^{ab}
ASG, 200	107.54 ± 10.36	130.02 ± 2.94 ^b	142.52 ± 11.17 ^{ab}
EST	99.64 ± 0.59 ^a	84.91 ± 3.93 ^b	85.15 ± 3.36 ^b
GABA, 100	140.74 ± 2.02 ^c	150.19 ± 4.00 ^b	121.23 ± 55.02 ^{ab}
GABA, 200	67.52 ± 8.34 ^f	89.63 ± 2.74 ^b	95.02 ± 8.12 ^b
GBR, 100	172.55 ± 5.60 ^b	158.31 ± 3.05 ^b	135.77 ± 21.85 ^{ab}
GBR, 200	162.60 ± 8.44	123.16 ± 2.21 ^b	133.12 ± 22.12 ^{ab}
ORZ, 100	95.46 ± 4.65 ^{ef}	102.91 ± 3.01 ^b	95.67 ± 4.09 ^b
ORZ, 200	75.76 ± 2.11 ^{fg}	88.90 ± 1.71 ^b	84.32 ± 4.10 ^b
OVX	298.93 ± 6.09 ^a	374.09 ± 74.77 ^a	201.69 ± 4.15 ^a
REM, 100	178.39 ± 2.72 ^b	155.57 ± 3.59 ^b	142.05 ± 5.92 ^{ab}
REM, 200	121.67 ± 2.31 ^{cd}	116.70 ± 4.96 ^b	129.60 ± 28.66 ^{ab}
Sham	75.85 ± 2.08 ^{fg}	79.67 ± 11.34 ^b	80.36 ± 1.22 ^b

Notes: Values in the table represent the mean ± standard deviation for three readings (n = 3). Levels not connected by the same superscript letters within the same week are significantly different (P < 0.05).

Abbreviations: ASG, acylated steryl glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; IL-6, interleukin-6; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin.

target gene tagged to an 18-nucleotide universal forward sequence (Table 4).

cDNA synthesis

From each sample, 250 ng of RNA was reverse transcribed to complementary DNA (cDNA) using multiplex universal reverse primers. Reactions were performed following the protocols stated in the GenomeLab™ Start Kit (Beckman Coulter, Brea, CA, USA) using a thermal-cycler at 48°C for 1 minute; 37°C for 5 minutes; 42°C for 60 minutes; 95°C for 5 minutes, and holding at 4°C.

PCR amplification

The PCR was carried out in a reaction mixture containing the cDNA from the reverse transcription reaction product (9.3 µL), 200 nM forward universal primer set mix (2 µL), 25 mM MgCl₂ (4 µL), Thermo Start Taq DNA polymerase (0.7 µL), and 5 × PCR Master Mix buffer (4 µL). Amplification was done initially by denaturation at 95°C for 10 minutes, then by 35 two-step cycles of 94°C for 30 seconds and 55°C for 30 seconds, ending in a single extension cycle of 68°C for 1 minute in an XP Thermal Cycler.

GeXP multiplex data analysis

The GeXPS machine (Beckman Coulter, Brea, CA, USA) was used to separate PCR products based on size by capillary gel electrophoresis and to measure their dye signal strength

in arbitrary units (AU) of optical fluorescence, which is the fluorescent signal minus background. PCR products were diluted in water at a ratio of 2:8, and 1 µL of this solution was added to a 38.5 µL sample loading solution with 0.5 µL of DNA size standard 400.

Fragment analysis and gene expression signature analysis

Data were initially analyzed using the Fragment Analysis module of the gene expression machine before importing into a profiler software. Control genes were tested for result consistency and the *cyclophilin A* gene gave consistent results, so it was chosen for normalizing the data for all the genes of interest.

Statistical analysis

Data were expressed as mean ± standard deviation, and the means were compared using ANOVA (analysis of variance), while the differences between the means were determined via the Tukey–Kramer post-hoc test using JMP 10 statistical software (SAS Institute, Cary, NC, USA). Values for P < 0.05 were considered statistically significant.

Results

Body weight

There was no significant difference found in terms of weight between the groups just before the initiation of treatments and 2 weeks after the surgery; a steady increase in body

Table 2 Body weight (g) in OVX rats treated with GBR-phenolic compounds, ASG, GABA, ORZ, EST, or REM over a period of 8 weeks

Treatment groups (mg/kg)	Body weight(g)		
	Week 2	Week 4	Week 8
ASG, 100	264.66 ± 1.63 ^{abc}	282.50 ± 3.39 ^{abc}	322.50 ± 3.72 ^{bc}
ASG, 200	259.33 ± 6.05 ^{cd}	273.00 ± 4.56 ^{ef}	315.50 ± 5.16 ^c
OVX + EST	261.83 ± 2.85 ^{abc,d}	273.83 ± 4.57 ^{d,ef}	290.16 ± 4.53 ^d
GABA, 100	265.66 ± 2.25 ^{abc}	282.83 ± 3.97 ^{abc}	323.16 ± 3.71 ^b
GABA, 200	261.33 ± 5.12 ^{abc,d}	274.83 ± 4.53 ^{c,d,ef}	319.66 ± 3.14 ^{bc}
GBR, 100	264.50 ± 2.58 ^{abc,d}	282.83 ± 2.48 ^{abc}	322.66 ± 3.44 ^{bc}
GBR, 200	258.83 ± 5.94 ^{cd}	274.16 ± 3.65 ^{d,ef}	316.00 ± 3.03 ^{bc}
ORZ, 100	264.00 ± 1.78 ^{abc,d}	278.00 ± 6.81 ^{c,d,e}	321.83 ± 2.13 ^{bc}
ORZ, 200	260.00 ± 6.16 ^{b,c,d}	276.50 ± 3.61 ^{c,d,e}	317.50 ± 3.93 ^{bc}
OVX	264.00 ± 3.74 ^{abc,d}	288.83 ± 2.48 ^a	336.66 ± 4.54 ^a
REM, 10	260.83 ± 1.72 ^{abc,d}	278.33 ± 1.86 ^{b,c,d,e}	320.00 ± 3.57 ^{bc}
REM, 20	259.00 ± 3.89 ^{cd}	274.16 ± 3.65 ^{d,ef}	316.00 ± 3.03 ^{bc}
Sham	256.33 ± 5.85 ^d	267.66 ± 4.13 ^f	284.66 ± 3.88 ^d

Notes: Values in the table represent the mean ± standard deviation (n = 6). Levels not connected by the same superscript letters within the same week are significantly different (P < 0.05).

Abbreviations: ASG, acylated steryl glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin.

weight was observed in all the groups from week 2 to week 8 (Table 2). A slight increase in body weight, though not significant ($P > 0.05$) in all the OVX treated groups was observed at week 2 after treatment, compared to the sham non-OVX group (Table 2). A significant increase in weight was observed in the OVX non-treated group compared to sham and all the OVX treated groups at weeks 4 and 8 after treatment ($P < 0.05$), respectively (Table 2). No significant difference ($P > 0.05$) between the EST 0.2 mg/kg treated group and the sham non-OVX group was found at week 8 of treatment (Table 2).

Serum IL-6 and osteocalcin ELISA

Serum IL-6 increased significantly at weeks 2, 4, and 8 in the OVX-non-treated group compared to the sham and other OVX treated groups ($P < 0.05$) as shown in Table 1. IL-6 concentration decreased significantly in groups treated with GABA 200 mg/kg; the sham non-OVX group; and groups treated with EST, ORZ, and ASG at 200 mg/kg; and REM 20 mg/kg ($P < 0.05$) at weeks 2, 4, and 8 (Table 1). The osteocalcin level significantly increased in groups treated with EST and ORZ at 200 mg/kg and the sham non-OVX group compared to the other treated groups, at week 2 ($P < 0.05$). The concentration also significantly increased ($P < 0.05$) at weeks 4 and 8 after treatment in groups treated with EST, REM 20 mg/kg, ASG, ORZ, GABA, and GBR at 200 mg/kg (Table 3).

Table 3 Serum osteocalcin concentration in OVX rats treated with GBR-phenolic compounds, ASG, GABA, ORZ, EST, or REM over a period of 8 weeks

Treatment groups (mg/kg)	Osteocalcin concentration (ng/mL)		
	Week 2	Week 4	Week 8
ASG, 100	161.65 ± 12.22 ^e	176.21 ± 4.25 ^{c,d,e}	154.62 ± 2.40 ^{b,c}
ASG, 200	201.84 ± 4.19 ^{a,b,c}	184.23 ± 7.89 ^{a,b,c,d}	172.03 ± 3.66 ^{a,b}
EST	204.04 ± 4.60 ^{ab}	189.62 ± 11.66 ^{a,b,c}	184.09 ± 4.07 ^{a,b}
GABA, 100	190.37 ± 1.54 ^{b,c,d,e}	180.56 ± 2.15 ^{b,c,d}	131.68 ± 7.83 ^c
GABA, 200	200.06 ± 6.53 ^{ab,c}	203.45 ± 3.82 ^{ab}	163.96 ± 5.67 ^{a,b,c}
GBR, 100	184.21 ± 2.20 ^{b,c,d}	160.12 ± 12.24 ^{d,e}	153.50 ± 7.74 ^{b,c}
GBR, 200	199.89 ± 0.33 ^{ab,c}	193.29 ± 6.44 ^{a,b,c}	160.65 ± 1.24 ^{b,c}
ORZ, 100	191.56 ± 8.62 ^{b,c,d}	170.24 ± 1.49 ^{c,d,e}	147.34 ± 3.02 ^{b,c}
ORZ, 200	221.24 ± 4.50 ^a	209.66 ± 3.35 ^a	203.18 ± 29.64 ^a
OVX	184.17 ± 8.23 ^{b,c,d,e}	173.57 ± 5.80 ^{c,d,e}	169.92 ± 13.54 ^{a,b,c}
REM, 10	178.39 ± 4.33 ^{c,d,e}	182.66 ± 3.24 ^{b,c,d}	162.09 ± 3.66 ^{b,c}
REM, 20	174.72 ± 7.28 ^{d,e}	191.10 ± 2.34 ^{a,b,c}	178.93 ± 0.92 ^{a,b}
Sham	218.84 ± 3.96 ^a	151.55 ± 7.42 ^e	179.91 ± 5.04 ^{a,b}

Notes: Values in the table represent the mean ± standard deviation ($n = 3$). Levels not connected by the same superscript letters within the same week are significantly different ($P < 0.05$).

Abbreviations: ASG, acylated steryl glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin.

Gene expression

Results for the primer sequence generated from the National Center for Biotechnology Information (NCBI) with the genes' descriptions, their accession numbers, and product sizes are described in Table 1. *ATP synthase*, *cyclophilin A*, *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, and *Actb* serve as the housekeeping genes, with *KANr* as the internal control, while the other seven genes are related to bone formation/metabolism as shown in Table 4. Results for the relative expression of the seven genes are described in Figures 1–7.

Expression of calcitonin receptor gene (CGRP)

The group treated with GABA at 100 mg/kg gave the highest significant upregulation ($P < 0.05$) of *CGRP* compared to the OVX-non-treated group, non-OVX, non-treated (sham) group, and groups treated with ASG, ORZ, and GBR at 100 and 200 mg/kg, EST at 0.2 mg/kg, and GABA at 200 mg/kg (Figure 1). The groups treated with REM at 20 mg/kg and ASG at 200 mg/kg were significantly upregulated ($P < 0.05$) compared to OVX non-treated group and groups treated with ORZ at 100 and 200mg/kg, ASG at 100 mg/kg, GBR at 100 mg/kg, and EST at 0.2 mg/kg (Figure 1).

Expression of BMP-2 gene

Group treated with GABA at 200 mg/kg showed the highest significant upregulation in the expression of *BMP-2* ($P < 0.05$) compared to the OVX-non-treated group, non-OVX non-treated (sham), and groups treated with ASG at 100 and 200 mg/kg, ORZ at 200 mg/kg, REM at 20 mg/kg, GBR at 200 mg/kg, and EST at 0.2 mg/kg (Figure 2).

Expression of SPARC gene

The group treated with GABA at 100 mg/kg showed significant ($P < 0.05$) expression of the *SPARC* gene compared to the OVX-non-treated group, the non-OVX non-treated (sham), and groups treated with REM at 10 and 20 mg/kg, EST at 0.2 mg/kg, ASG at 100 and 200 mg/kg, GBR at 100 mg/kg, GABA at 200 mg/kg, and ORZ at 100 and 200 mg/kg (Figure 3). The group treated with GBR at 200 mg/kg was significantly different ($P < 0.05$) to the OVX-non-treated group and groups treated with GBR at 100 mg/kg, REM at 10 and 20 mg/kg, EST, ASG at 100 mg/kg, and ORZ at 200 mg/kg. The group treated with ORZ at 100 mg/kg showed significant upregulation of the *SPARC* gene ($P < 0.05$) compared to groups treated with ASG at 100 mg/kg, REM at 10 and 20 mg/kg, and EST at 0.2 mg/kg (Figure 3). The group treated with ASG at 200 mg/kg was upregulated

Table 4 Gene description, accession number, primer sequence, and the product size of the selected genes

Gene description	Accession no	Primer sequence	Product size	Comment
ATP synthase	NM_138883	F: 5'-AGGTGACACTATAGAATACTCTCTGAGTTAAAGACAGTGCTGA-3' R: 5'-GTACGACTCACTATAGGGAACAATCATCCCACCCATGAT-3'	138	Housekeeping gene
Peptidylprolyl isomerase A (cyclophilin A)	NM_017101	F: 5'-AGGTGACACTATAGAATATTCTGTAGCTCAGGAGAGCA-3' R: 5'-GTACGACTCACTATAGGGATTGAAGGGGAATGAGGAAAA-3'	145	Housekeeping gene
CGRP	NM_001034015	F: 5'-AGGTGACACTATAGAATAACCTGGTGGCAGCGG-3' R: 5'-GTACGACTCACTATAGGGATGACGATCAGAGTGTGAAGATAGA-3'	157	Gene related to osteoblastic activity
BMP-2	NM_017178	F: 5'-TGA ACA CAG CTG GTC TCAGG-3' R: 5'-TTA AGA CGC TTC CGC TGTTT-3'	167	Gene related to osteoblastic activity
SPARC	NM_012656	F: 5'-AGGTGACACTATAGAATAGACCTTCGACTCTTCCTGCC-3' R: 5'-GTACGACTCACTATAGGGAGGAATTCGGTCAGCTCAGAA-3'	177	Gene related to osteoblastic activity
RUNX-2	NM_053470	F: 5'-GCC GGG AAT GAT GAG AACTA-3' R: 5'-GGA CCG TCC ACT GTC ACTTT-3'	187	Gene related to osteoblastic activity
OSX	NM_181374	F: 5'-GCT GCC TAC TTA CCC GTCTG-3' R: 5'-GTT GCC CAC TAT TGC CAACT-3'	207	Gene related to osteoblastic activity
Postn	NM_001108550	F: 5'-AGGTGACACTATAGAATAGCCACGACCACTCAACACTA-3' R: 5'-GTACGACTCACTATAGGGACTGGGGTGTGACAGGTTG-3'	217	Gene related to osteoblastic activity
Col1 &2	NM_053356	F: 5'-AGGTGACACTATAGAATACGAGACCCCTTCTCACTCCTG-3' R: 5'-GTACGACTCACTATAGGGACTGGGGTGTGACAGGTTG-3'	237	Gene related to osteoblastic activity
GAPDH	NM_017008	F: 5'-GTG GAC CTC ATG GCC TACAT-3' R: 5'-TGT GAG GGA GAT GCT CAGTG-3'	271	Housekeeping gene
Actb	NM_031144	F: 5'-AGGTGACACTATAGAATAGGCATCCTGACCCTGAAGTA-3' R: 5'-GTACGACTCACTATAGGGAAGACGCAGGATGGCATGAG-3'	384	Housekeeping gene
KANr				Internal control

Abbreviations: Actb, actin beta; ATP, adenosine triphosphate; BMP-2, bone morphogenic protein-2; Col1 &2, collagen 1 &2; CGRP, calcitonin receptor; SPARC, secreted protein acidic and rich in cysteine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Runx-2, runt-related transcription factor 2; OSX, osterix; Postn, periostin, osteoblast specific factor.

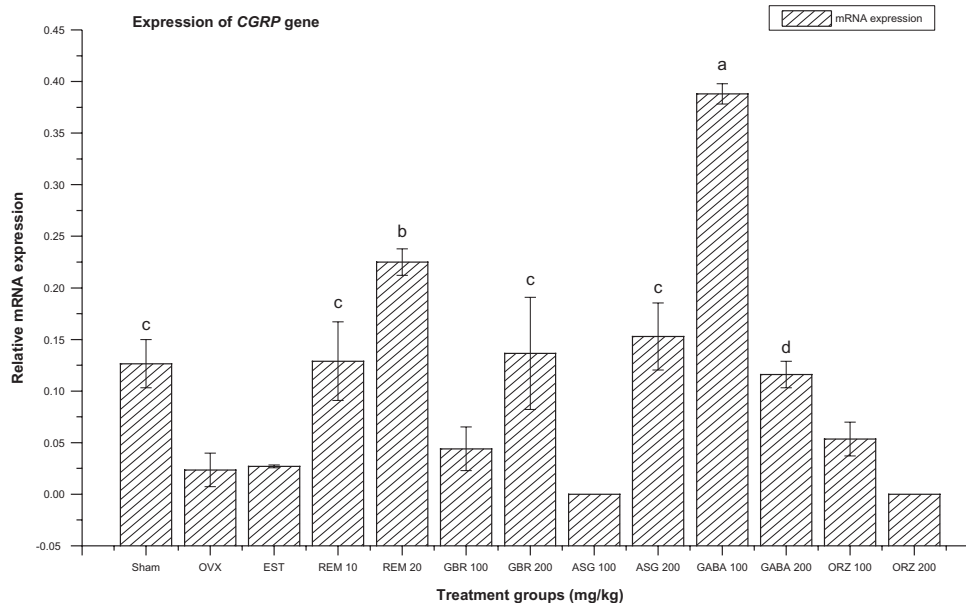


Figure 1 Relative mRNA expression of CGRP gene in OVX rats treated with EST, REM, GBR, GABA and ORZ in different doses compared to sham and OVX non-treated group.

Notes: Treatment groups were expressed in (mg/kg) for three readings, levels not connected by same letter are significantly different ($P < 0.05$). ^aGroup treated with GABA at the dose of 100 mg/kg shows the highest significant upregulation of CGRP gene compared to sham, OVX-non treated and all other treatment groups ($P < 0.001$); ^bgroup treated with REM 20 mg/kg shows a significant upregulation compared to sham, OVX non-treated, EST, ORZ, GBR, and REM 10 mg/kg treated groups ($P < 0.001$); ^cgroups treated with REM 10 mg/kg, GBR and ASG 200 mg/kg showed an upregulation equal to that of sham (non-OVX group) and greater than that of OVX non-treated group ($P < 0.05$); ^dgroup treated with GABA 200 mg/kg shows a significant upregulation compared to OVX non-treated group groups treated with ORZ 200 mg/kg, ASG 100 mg/kg, and EST 0.2 mg/kg treated groups ($P < 0.05$).

Abbreviations: ASG, acylated steryl glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin; mRNA, messenger ribonucleic acid; CGRP, calcitonin receptor.

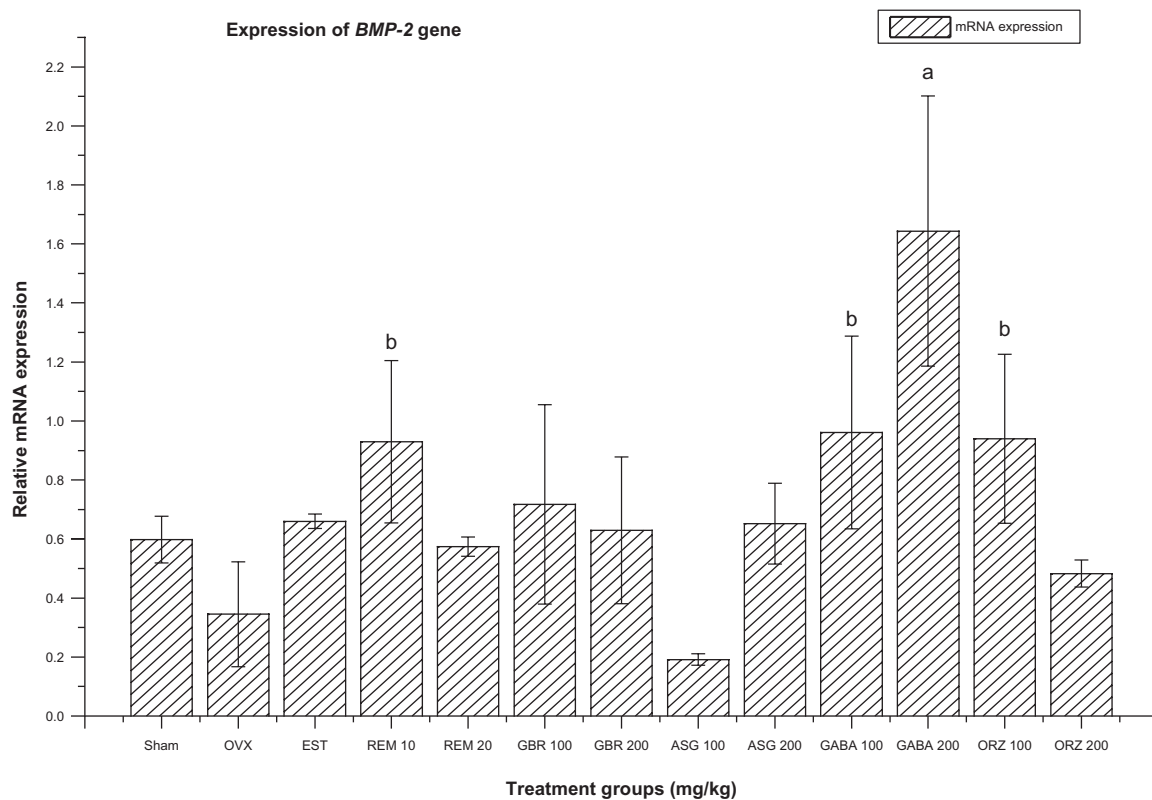


Figure 2 Relative mRNA expression of *BMP-2* gene in OVX rats treated with EST, REM, GBR, GABA and ORZ in different doses compared to sham and OVX non-treated groups.

Notes: Treatment groups were expressed in (mg/kg) for three readings, levels not connected by same letter are significantly different ($P < 0.05$). *GABA 200 mg/kg treated group had the highest expression of *BMP-2* gene compared to OVX non-treated, sham non-OVX and all the other treated groups ($P < 0.05$); ^bgroup treated with REM 10 mg/kg, GABA and ORZ 100 mg/kg showed a significant upregulation compared to sham, OVX non-treated, EST 0.2 mg/kg, ORZ 200, GBR and ASG 100 and 200 mg/kg and REM 20 mg/kg treated groups ($P < 0.001$).

Abbreviations: ASG, acylated steryl glucosides; BMP, bone morphogenic protein; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin; mRNA, messenger ribonucleic acid.

($P < 0.05$) when compared to the OVX-non-treated group, groups treated with REM at 10 and 20 mg/kg, EST, and ASG at 100 mg/kg, while the OVX-non-treated group and groups treated with EST 0.2 mg/kg, ASG 100 mg/kg, and GBR 100 mg/kg were downregulated compared to the sham (non-OVX, non-treated) groups (Figure 3).

Expression of *RUNX-2* gene

The group treated with ORZ at 200 mg/kg was highly upregulated ($P < 0.05$) in the expression of the *RUNX-2* gene compared to the OVX-non-treated group, sham, EST, and those groups treated with ORZ at 100 mg/kg, GABA at 100 and 200 mg/kg, GBR at 100 and 200 mg/kg, REM at 100 and 200 mg/kg, and ASG at 100 and 200 mg/kg (Figure 4). The group treated with ASG at 100 mg/kg also differed significantly ($P < 0.05$) from the OVX-non-treated group and the groups treated with GBR at 100 and 200 mg/kg, REM at 10 and 20 mg/kg, ASG at 200 mg/kg, and GABA and ORZ at 100 mg/kg (Figure 4). The group treated with EST at 0.2 mg/kg showed significant upregulation compared with the OVX-non-treated group, ORZ at 100 mg/kg, REM at

10 mg/kg, GBR at 200 mg/kg, and ASG at 200 mg/kg ($P < 0.05$). The OVX-non-treated group and groups treated with ORZ and GABA at 100 mg/kg, GBR and ASG at 200 mg/kg, and REM at 10 mg/kg were significantly downregulated compared to the non-OVX, non-treated (sham) group ($P < 0.05$). The GABA 200 mg/kg treated group was also upregulated and significantly different to the OVX-non-treated group, as well as groups treated with GABA at 100 mg/kg, GBR and ASG at 200 mg/kg, and REM at 10 and 20 mg/kg. Lastly, the groups treated with GBR at 100 mg/kg and REM at 20 mg/kg were significantly upregulated ($P < 0.05$) compared to the group treated with ORZ at 100 mg/kg (Figure 4).

Expression of *OSX* gene

The *OSX* gene expression was significantly upregulated ($P < 0.05$) in the group treated with ORZ at 200 mg/kg when compared to the OVX-non-treated group, ORZ at 100 mg/kg group, sham, and groups treated with GBR at 100 and 200 mg/kg, REM at 10 and 20 mg/kg, and GABA at 100 and 200 mg/kg (Figure 5). The group treated with GABA at 200 mg/kg showed a significant upregulation compared

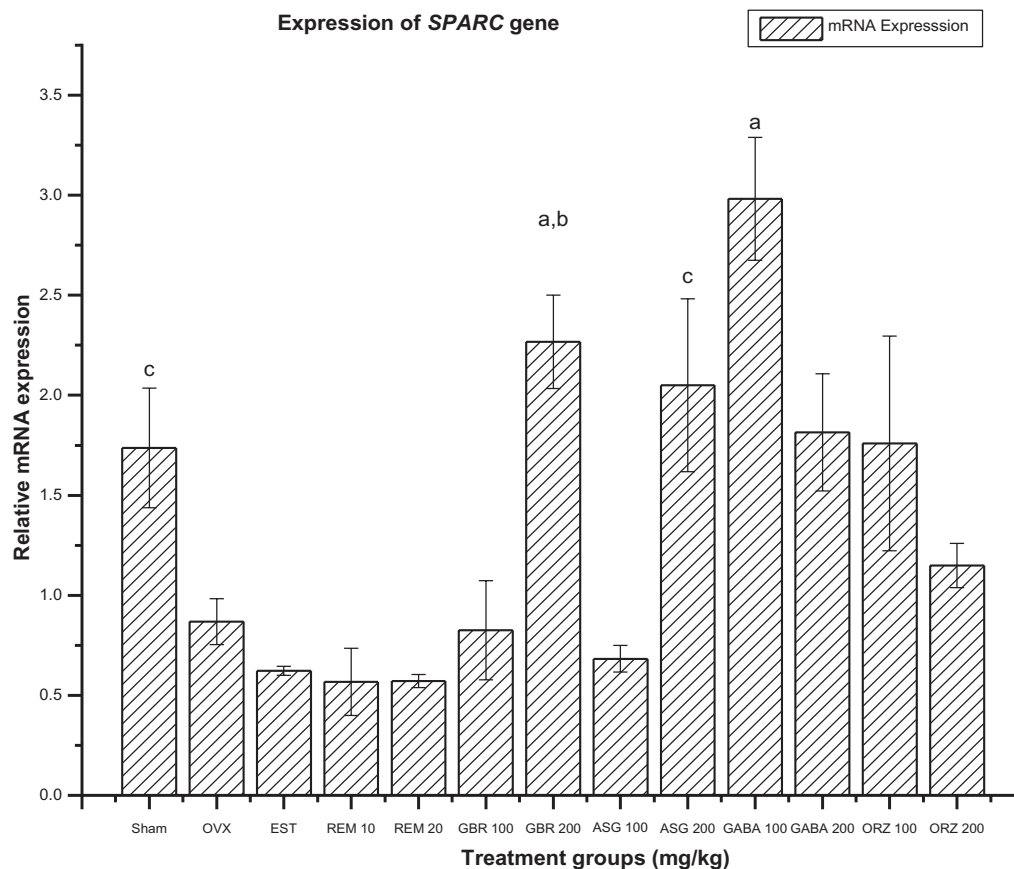


Figure 3 Relative mRNA expression of *SPARC* gene in OVX rats treated with EST, REM, GBR, GABA and ORZ in different doses compared to sham and OVX non-treated group.

Notes: Treatment groups were expressed in (mg/kg) for three readings, levels not connected by same letter are significantly different ($P < 0.05$). ^aGroup treated with GABA 200 mg/kg gave the highest in term of the expression of *SPARC* gene compared to OVX non-treated, sham non-OVX and all the other treated groups ($P < 0.0001$); ^bgroup treated with GBR 200 mg/kg gives a significant upregulation of *SPARC* gene difference compared to OVX non-treated, sham non-OVX and groups treated with EST 0.2 mg/kg, REM 10 and 20 mg/kg, GBR and ASG 100 mg/kg and ORZ 200 mg/kg ($P < 0.05$); ^cno significant difference ($P > 0.05$) between the sham non OVX group and groups treated with ASG 200 mg/kg.

Abbreviations: ASG, acylated sterol glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin; mRNA, messenger ribonucleic acid; *SPARC*, secreted protein acidic and rich in cysteine.

to the OVX-non-treated group and the groups treated with ASG at 100 and 200 mg/kg, ORZ at 100 mg/kg, EST at 0.2 mg/kg, REM at 10 mg/kg, and GBR at 200 mg/kg. The group treated with ASG at 100 mg/kg was significantly ($P < 0.05$) downregulated compared to the sham (non-OVX, non-treated) group, and groups treated with REM at 20 mg/kg and GABA at 100 mg/kg (Figure 5).

Expression of *Postn* gene

The OVX-non-treated group, and groups treated with ASG at 100 and 200 mg/kg, GBR at 100 and 200 mg/kg, REM at 10 and 20 mg/kg, ORZ at 100 and 200 mg/kg, GABA at 100 and 200 mg/kg, and EST 0.2 mg/kg were significantly downregulated in the expression of the *Postn* gene compared to the sham (non-OVX, non-treated) group (Figure 6). The group treated with ORZ at 200 mg/kg showed upregulation ($P < 0.05$) compared to the OVX-non-treated group and groups treated with ASG at 100 and 200 mg/kg, GBR at 100

and 200 mg/kg, REM at 10 and 20 mg/kg, GABA at 100 and 200 mg/kg, EST at 0.2 mg/kg, and ORZ at 100 mg/kg. The groups treated with GABA at 100 and 200 mg/kg, ORZ at 100 mg/kg, ORZ at 100 mg/kg and REM at 20 mg/kg showed significant upregulation compared to the group treated with ASG at 100 mg/kg (Figure 6).

Expression of *collagen 1&2 (coll&2)* gene

The OVX-non-treated group, and groups treated with GABA at 100 and 200 mg/kg, REM at 10 and 20 mg/kg, ASG at 200 mg/kg, EST at 0.2 mg/kg, and ORZ at 200 mg/kg were significantly downregulated ($P < 0.05$) in the expression of *coll1&2* compared to the sham group (non-OVX, non-treated). The group treated with ORZ at 100 mg/kg was upregulated compared to the OVX-non-treated group, and groups treated with GABA at 200 mg/kg, REM at 10 and 20 mg/kg, GBR at 100 mg/kg, ASG at 200 mg/kg, EST at 0.2 mg/kg, and ORZ at 200 mg/kg (Figure 7). The group

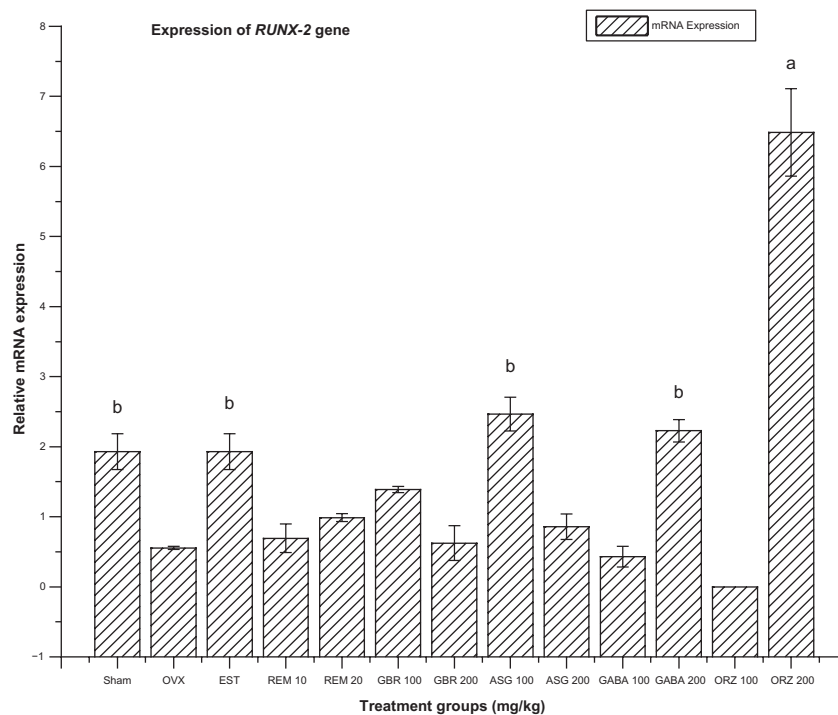


Figure 4 Relative mRNA expression of *RUNX-2* gene in OVX rats treated with EST, REM, GBR, GABA and ORZ in different doses compared to sham and OVX non-treated group.

Notes: Treatment groups were expressed in (mg/kg) for three readings, levels not connected by same letter are significantly different ($P < 0.05$). *Group treated with ORZ 200 mg/kg gave the highest and significant expression of *RUNX-2* gene compared to the other treatment groups ($P < 0.001$); ^bgroups treated with EST 0.2 mg/kg, ASG 100 mg/kg gave almost the same expression in term of *RUNX-2* gene compared to sham ($P > 0.05$) and significantly higher compared to other groups ($P < 0.05$).

Abbreviations: ASG, acylated steryl glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin; mRNA, messenger ribonucleic acid; *RUNX-2*, runt-related transcription factor 2.

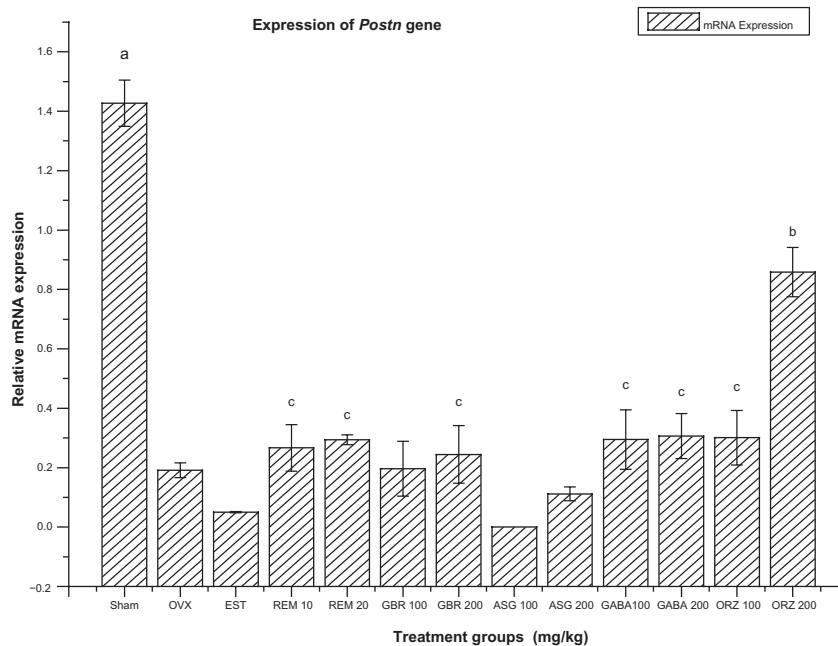


Figure 5 Relative mRNA expression of *Postn* gene in OVX rats treated with EST, REM, GBR, GABA and ORZ in different doses compared to sham and OVX non-treated group.

Notes: Treatment groups were expressed in (mg/kg) for three readings, levels not connected by same letter are significantly different ($P < 0.05$). *Sham non-OVX group gave the highest expression of *Postn* gene compared to all treatment groups ($P < 0.001$); ^bgroup treated with ORZ at 200 mg/kg gives the highest significant upregulation of *Postn* gene compared to all other treatment groups ($P < 0.001$); ^cno significant difference was observed when comparing the treatments in these groups ($P > 0.05$); But significantly higher in terms of expression of *Postn* compared to OVX non-treated group ($P < 0.05$).

Abbreviations: ASG, acylated steryl glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin; mRNA, messenger RNA; *Postn*, periostin, osteoblast specific factor.

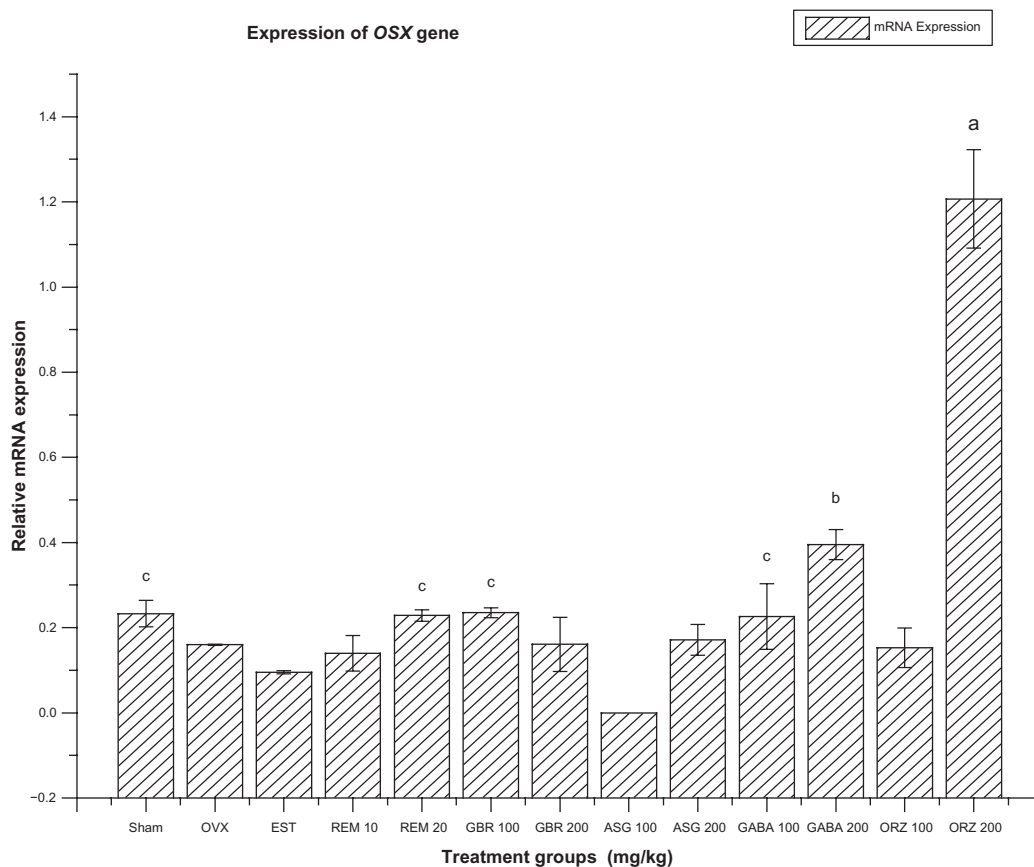


Figure 6 Relative mRNA expression of OSX gene in OVX rats treated with EST, REM, GBR, GABA and ORZ in different doses compared to sham and OVX non-treated group.

Notes: Treatment groups were expressed in (mg/kg) for three readings, levels not connected by same letter are significantly different ($P < 0.05$). ^aGroup treated with ORZ 200 mg/kg gave the highest expression of OSX gene compared to sham, OVX non-treated, and all other treatment groups ($P < 0.001$); ^bGABA 200 mg/kg treated group gave a significant high expression of OSX gene compared to other treatment groups ($P < 0.05$); ^cno significant difference ($P > 0.05$) between the sham non-OVX group compared to groups treated with REM 20 mg/kg, GBR and GABA 100 mg/kg.

Abbreviations: ASG, acylated sterol glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin; mRNA, messenger ribonucleic acid; OSX, osterix.

treated with ASG at 100 mg/kg was significantly upregulated ($P < 0.05$) compared to groups treated with GBR at 100 mg/kg, REM at 10 and 20 mg/kg, and EST at 0.2 mg/kg. The OVX-non-treated group and groups treated with GBR at 100 mg/kg, REM at 10 and 20 mg/kg, EST at 0.2 mg/kg, and GABA at 200 mg/kg were significantly downregulated ($P < 0.05$) compared to groups treated with GABA at 100 mg/kg and ORZ at 200 mg/kg. The group treated with ASG at 200 mg/kg differed significantly ($P < 0.05$) to the group treated with EST at 0.2 mg/kg (Figure 7).

Discussion

This study, examines the relative expression of genes related to bone metabolism, osteocalcin, and IL-6 levels in OVX rats treated with GBR-phenolics, ORZ, ASG, and GABA extracted from GBR, in comparison to groups treated with EST and REM. Total body weight increased in the OVX rats compared to the sham non-OVX group; as shown in Table 1,

there was a difference of 52 g between the sham and the OVX group. Ovariectomies have been reported to increase body mass during the first 3 weeks after surgery.³³ Decreases in ovarian hormones in rats are associated with increased food intake and decreased motor activity, which leads to increased body mass.^{34,35} Treatment with EST and GBR bioactives for 8 weeks significantly reduced the increase in body weight induced by ovariectomy with EST given the most significant effect; this shows that GBR and its bioactive compounds has weaker estrogenic effect. Our data show that GABA and ORZ at 200 mg/kg decreased serum IL-6 and increased osteocalcin concentration. ORZ, a mixture of ferulic acid esters of triterpene alcohols, is known to possess powerful antioxidant and anti-inflammatory effects.³⁶⁻³⁸ In this study, we found that the calcitonin gene was upregulated in groups treated with GABA at 100 mg/kg when compared to the other treatment groups; likewise *BMP-2* and *SPARC* were both upregulated in groups treated with GABA at 200 and

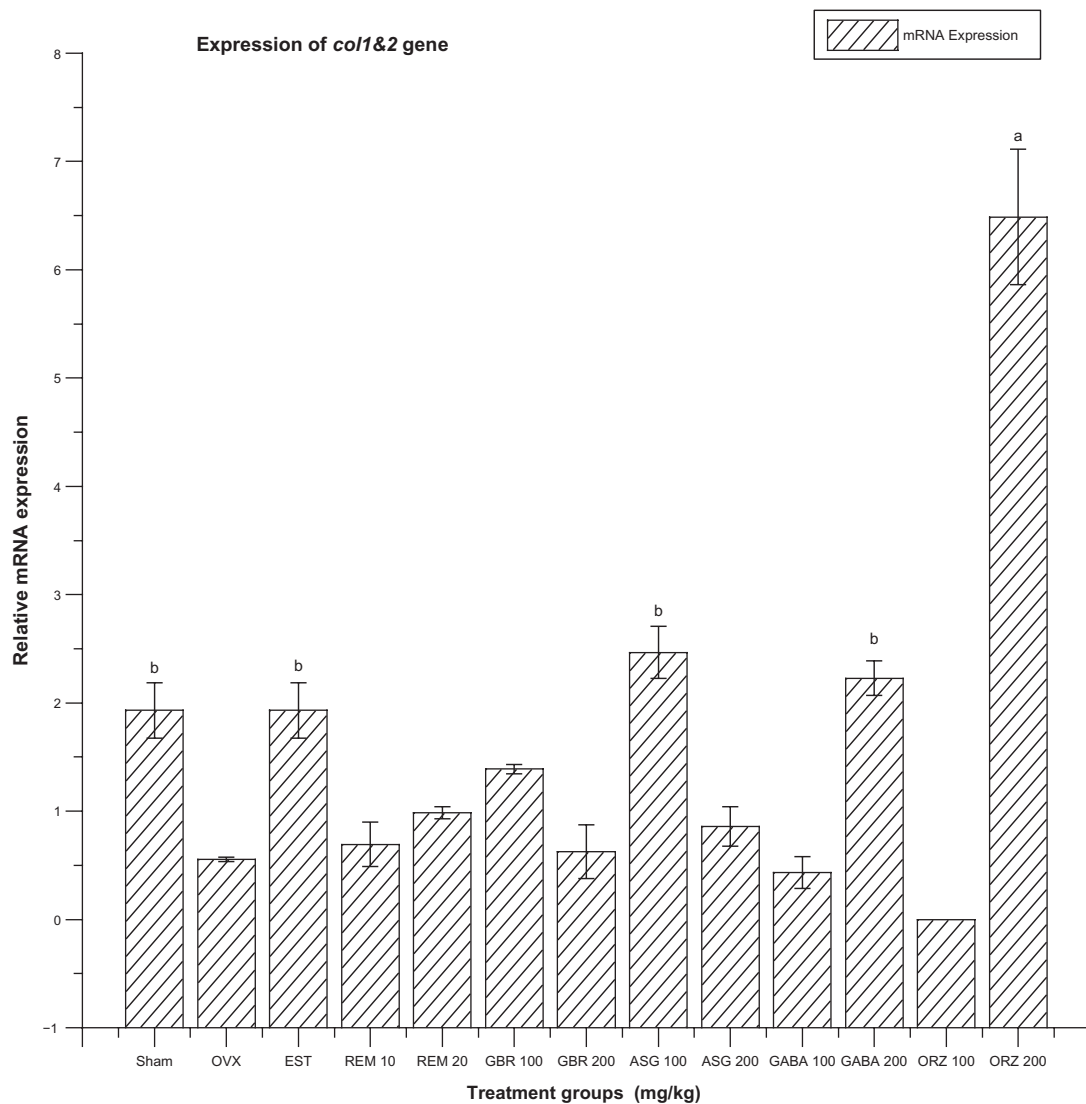


Figure 7 Relative mRNA expression of *col1&2* gene in OVX rats treated with EST, REM, GBR, GABA and ORZ in different doses compared to sham and OVX non-treated group.

Notes: Treatment groups were expressed in (mg/kg) for three readings, levels not connected by same letter are significantly different ($P < 0.05$). ^aGroup treated with ORZ 200 mg/kg gave the highest significant expression of *col1&2* gene compared to other treatment groups ($P < 0.001$); ^bgroup treated with ORZ 200 mg/kg gave the highest significant expression of *col1&2* gene compared to OVX non-treated, sham, and all the other treatment groups ($P < 0.0001$); ^cno significant difference ($P > 0.05$) between the sham non-OVX group compared to groups treated with EST 0.2 mg/kg, ASG 100 mg/kg and GABA 200 mg/kg.

Abbreviations: ASG, acylated steryl glucosides; EST, estrogen; GABA, gamma-amino butyric acid; GBR, phenolic extracts from germinated brown rice; ORZ, gamma-oryzanol; OVX, ovariectomized; REM, remifemin; mRNA, messenger ribonucleic acid; *col1&2*, collagen 1&2.

100 mg/kg, respectively. The neuroprotective, antioxidant, and memory enhancement properties attributed to the high content of GABA in GBR have been documented in other studies.³⁹⁻⁴¹ It has been reported that GABA increases the level of human growth hormone, reduces fat mass, and increases bone mass density.⁴²⁻⁴⁴ The GABA receptor antagonist picrotoxin inhibits bone formation and decreases calcium content in rats at various growth sites of the skull.⁴⁵ Similarly, GABA may play a role in mechanisms associated with cellular proliferation, differentiation, and development through GABA_B receptors (GABA_BR) expressed in cultured osteoblasts.⁴⁶ In the same vein, Takahata et al⁴⁷ showed

that functional GABA_BR is predominantly expressed by osteoblasts rather than osteoclasts during bone remodeling as well as in skeletogenesis, and GABAergic signaling could be a novel target for the treatment of bone diseases. The upregulation of *BMP-2*, *SPARC*, and *osteocalcin* genes by GABA therefore may be attributed to the stimulation of GABA_B receptors in the osteoblasts, which initiates the GABAergic signaling that in turn stimulates the expression of *BMP-2*, *SPARC*, and *osteocalcin*. It has been reported that functional GABA_BR are predominantly expressed by osteoblasts rather than osteoclasts to regulate cellular maturation related to *BMP-2* expression.⁴⁷ Groups treated with ORZ at

200 and 100 mg/kg gave the highest upregulation in terms of expression of *OSX*, *Postn*, *RUNX-2*, and *coll&2* compared to other treated and non-treated groups. ORZ is known for its anticholesteremic, antioxidant, anti-inflammatory, and anti-platelet aggregation properties.^{48–50} Ovariectomies have been shown to induce oxidative stress and impair bone antioxidant systems in adult rats.⁵¹ The upregulation of these bone formation genes by ORZ may be attributed to their anti-inflammatory and antioxidant effects. Decreases in EST level in osteoporosis have been linked to upregulation of osteoclastogenesis through the activation of receptor activator of nuclear factor kappa-B ligand (RANKL) and a decrease in osteoprotegerin production by osteoblasts, as well as increased expression of cytokines such as tumor necrosis factor alpha, IL-1, and IL-6, in osteoblasts.^{52–54} ORZ has been shown to have an upregulatory effect on antioxidant genes.⁵⁵ In another study, nicotine was linked to oxidative stress which in turn led to downregulation of the expression of bone formation genes such as *RUNX-2*.⁵⁶

Conclusion

GABA-treated groups had the highest expression of the *calcitonin* gene, *BMP-2*, and *SPARC* compared to groups treated with GBR-phenolics, ORZ, ASG, EST, or REM. This is possibly due to the activation of GABA_B-receptors. ORZ had the highest upregulation of the expression of *RUNX-2*, *Osx*, *Postn*, and *coll&2* than the other groups due to its antioxidative, as well as anti-inflammatory effects. More studies are underway to evaluate the effects of these compounds on GABA receptors, and to elucidate the effect of the combination of these bioactives on the studied genes, to properly ascertain their clinical relevance in the management of osteoporosis and other related diseases.

Acknowledgments/disclosure

We wish to thank Padiberas National (BERNAS) rice company in Sri Tiram Jaya, Malaysia for funding this research. We also wish to acknowledge Dr Ibrahim Anka for his guidance on the statistical analysis. The authors report no conflicts of interest in this work.

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