Cytotoxic and Antioxidant Effects of the Water Extract of the Traditional Chinese Herb Gusuibu (*Drynaria fortunei*) on Rat Osteoblasts

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Background and purpose: Gusuibu (*Drynaria fortunei*) is a traditional Chinese herb that has been claimed to have therapeutic effects on bone healing; however, a clinical mechanism responsible for this effect has not been identified. This study evaluated the cytotoxic and antioxidant effects of the water extract of gusuibu (WEGSB) on rat osteoblasts.

Materials and methods: Osteoblasts were prepared from neonatal Wistar rat calvarias and treated with WEGSB. Cell viability and alkaline phosphatase activity were determined. Intracellular reactive oxygen species were detected using the dye 2',7'-dichlorofluorescin, and mitochondrial membrane potential was detected using the dye 3,3'-dihexyloxacarbocyanine iodide and flow cytometry.

Results: WEGSB at 1 and 10 μg/mL was not cytotoxic to rat osteoblasts, but WEGSB at 100 μg/mL reduced cell viability and alkaline phosphatase activity in a time-dependent manner. Although WEGSB and hydrogen peroxide did not affect the mitochondrial membrane potential of rat osteoblasts, combined treatment with WEGSB (100 μg/mL) and hydrogen peroxide lowered the membrane potential of mitochondria and resulted in cell death. The basal level of intracellular reactive oxygen species in rat osteoblasts was significantly suppressed by WEGSB at 10 to 100 μg/mL. WEGSB (10 μg/mL)specifically inhibited hydrogen peroxide-induced oxidative stress without an effect on nitric oxide-induced stress. Hydrogen peroxide caused concentration-dependent death of rat osteoblasts, but WEGSB significantly protected cells from hydrogen peroxide-induced death.

Conclusion: This study has shown that WEGSB at 10 μg/mL is not cytotoxic to rat osteoblasts *in vitro*, and also that the extract at 10 μg/μL has an antioxidant effect on these cells. The antioxidant activity of WEGSB can protect rat osteoblasts from hydrogen peroxide-induced death and may promote bone recovery under similar pathologic conditions.

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Key words:
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Inflammation is a common response to tissue injury. Acute or chronic inflammation can lead to serious bone diseases such as rheumatoid arthritis, ankylosing spondylitis, and inflammation-induced osteoporosis [1–4]. During inflammation, immune cells produce great amounts of reactive oxygen species (ROS). As the mediators of inflammatory cytokine effects, ROS play an important role in the pathophysiology of inflammation-induced tissue injury [5, 6]. Hydrogen peroxide and nitric oxide are two critical ROS involved in inflammatory lesions [7–9].

Osteoblasts play a critical role in bone remodeling and healing [10]. Previous studies revealed that hydrogen peroxide and nitric oxide, produced by osteoblasts

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or activated mononuclear phagocytes, suppress osteoblast metabolism and viability [11–13]. This suppression results in the delay of bone fracture healing and leads to inflammation-induced bone resorption, osteopenia, or osteoporosis [14–16]. Therefore, decreasing the levels of intracellular ROS will promote the recovery of bone defects.

Several traditional Chinese herbs have been shown to have anti-inflammatory activity [17–21]. Extracts of certain traditional Chinese herbs including *Cynanchum wilfordii*, *Scutellaria baicalensis Georgi*, and *Tinospora tuberculata* can scavenge hydrogen peroxide or nitric oxide to reduce oxidative stress induced by these two oxidants [22–24]. In animal studies, several traditional Chinese herbs including unkei-to, hachimi-jio-gan, juzen-taiho-to, ru-yih-jin-huang-saan, jie-guu-saan, and chujo-to have been reported to have therapeutic effects on osteoporosis and bone fracture [25–28]. Gusuibu (GSB), including families *Polypodiaceae* and *Dayaliacea*, are traditional Chinese herbs and widely used as civilian medicines for the therapy of bone fractures. Ma et al reported that *Drynaria baronii*, one species of GSB, was able to promote calcification of the cultivated chick embryo primodium and increase proteoglycan synthesis [29]. However, the effect of GSB on osteoblasts at the molecular level is unknown.

The elevation of intracellular oxidative stress that results from bone disease-induced acute inflammation is toxic to osteoblasts and harmful to bone healing. The present study was designed to investigate the cytotoxic and antioxidant effects of the aqueous extract of GSB, *Drynaria fortunei*, on rat calvarial osteoblasts by the assessment of cell viability, alkaline phophatase activity, mitochondrial membrane potential, and intracellular ROS.

Materials and Methods

The gusuibu used in this study was grown in Chentu, Syhchuan, China. Gusuibu was provided and identified by Dr. Wei-Lai Peng, a Chinese medicine doctor. Dry pieces of gusuibu rhizoma were ground into a fine powder. WEGSB was prepared by decocting 50 g gusuibu powder with 400 mL water for 1 hour. After filtration, the filtrate was frozen at –70°C and then concentrated into a dry powder using a freeze dryer FD24 (Kingmech, Taipei, Taiwan). The extract was stored at room temperature and protected from light and moisture.

Rat osteoblasts were prepared from 3-day-old Wistar rat calvarias following the sequential enzymatic digestion method described previously [30]. Primary osteoblasts were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in 250 mL polystyrene tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. WEGSB was dissolved in dimethylsulfoxide (DMSO). To avoid DMSO toxicity, the concentration of the solvent was less than 0.1% (vol/vol). Osteoblasts were treated with different concentrations of WEGSB for various time intervals in independent experiments.

Rat osteoblasts were treated with 1, 10, and 100 mg/mL WEGSB for 24, 48, and 72 hours, and cell viability and alkaline phosphatase activity were assayed to determine the cytotoxicity of WEGSB on these cells.

Osteoblast viability was determined by testing mitochondrial enzyme function according to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method as described previously [31]. Briefly, 10,000 rat osteoblasts were subcultured into a 96-well tissue culture cluster overnight. After WEGSB treatment, osteoblasts were washed with 1 X PBS (phosphate-buffered saline; 0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The medium was renewed with DMEM containing 0.5 mg/mL MTT, and cells were cultured for another 3 hours. After adding DMSO, the cell lysates were analyzed colorimetrically using an enzyme-linked immunosorbent assay (ELISA) reader (MRX_{TC}, Dynex Technology, Chantilly, VA, USA) at a wavelength of 570 nm.

Alkaline phosphatase activity was analyzed according to the colorimetric protocol provided in the Sigma Diagnostics Alkaline, Acid and Prostatic Acid Phosphatase kit (Sigma Diagnostics, Inc, St. Louis, MO, USA). Briefly, approximately 10,000 rat osteoblasts were subcultured in a 96-well tissue culture cluster overnight. After treatment, extraction buffer (0.05% Triton X-100, 2 mM $MgCl_2$) was added to the osteoblasts, and the extracts were used for alkaline phosphatase activity assay: *p*-nitrophenyl phosphate forms a yellow hydrolytic product, *p*-nitrophenol, detected using the ELISA reader at a wavelength of 410 nm.

The membrane potential of osteoblastic mitochondria was determined using the flow cytometric method described previously [32]. Osteoblasts were subcultured into a 12-well tissue culture cluster overnight and then treated with WEGSB and fluorescent dye 3,3'-dihexyloxacarbocyanine iodide for 4 hours. The relative fluorescence intensity in cells was quantified using a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA).

The intracellular ROS of rat osteoblasts were determined using the flow cytometric method as described previously [33]. Osteoblasts (5×10^5) were subcultured

in a 12-well tissue culture cluster overnight and then treated with WEGSB for 4 hours. An ROS-sensitive dye, 2',7'-dichlorofluorescin diacetate, was co-cultured with WEGSB to identify peroxide radicals in rat osteoblasts. The relative fluorescence intensity in cells was quantified using a flow cytometer.

Statistical analysis

The difference between control and drug-treated groups was evaluated by Student's *t*-test. A p value of less than 0.05 was considered statistically significant. The difference between drug-treated groups was considered to be statistically significant when the p value from the Duncan's Multiple Range test was less than 0.05.

Results

Exposure of rat osteoblasts to 1 and 10 mg/mL WEGSB for 24, 48, and 72 hours did not cause cell death (Table 1). However, WEGSB at 100 mg/mL led to 20 and 35% death in 48 hour- and 72 hour-treated osteoblasts, respectively.

Alkaline phosphatase activity was unchanged in rat osteoblasts exposed to 1 and 10 μg/mL WEGSB for 24, 48, and 72 hours (Table 2), but decreased by 17 and 30% in rat osteoblasts exposed to 100 μg/mL WEGSB for 48 and 72 hours.

Exposure of rat osteoblasts to 100 μg/mL WEGSB or hydrogen peroxide did not influence the membrane potential of mitochondria (Table 3). Combined treatment with WEGSB (100 μg/mL) and hydrogen peroxide significantly inhibited about 30% of mitochondrial membrane potential in rat osteoblasts.

WEGSB at 1 μg/mL did not affect the basal levels of intracellular ROS in rat osteoblasts (Fig. 1). Treatment with 10 and 100 μg/mL WEGSB significantly decreased intracellular ROS in rat osteoblasts, with 10 μg/mL resulting in a 35% ($p = 0.003$) decrease and 100 μg/mL resulting in a 50% decrease ($p = 0.001$). Hydrogen peroxide increased intracellular ROS in rat osteoblasts twofold and sodium nitroprusside, a nitric oxide donor, increased it fivefold (Table 4). Combined treatment with WEGSB (10 μg/mL) and hydrogen peroxide significantly blocked hydrogen peroxide-induced intracellular ROS. However, combined treatment with WEGSB (10 μg/mL) and sodium nitroprusside failed to reduce intracellular ROS induced by the nitric oxide donor.

Exposure of rat osteoblasts to 50, 100, 150, and 200 μM hydrogen peroxide resulted in the death of 13, 68, 85, and 87% of cells, respectively (Fig. 2). WEGSB at 10 μg/mL decreased this osteoblast death by 5, 29, 28, and 22%, respectively.

Discussion

Inflammation in response to injury of bone and soft tissue can be harmful to bone healing, or even result in more serious bone disease. For example, wounding of soft tissue generates inflammatory mediators leading to the delay of bone fracture healing [4]. Acute local

Table 1. Concentration- and time-dependent effects of water extract of gusuibu (WEGSB) on osteoblast viability

WEGSB (μg/mL)	Cell viability, % of control		
	24 hr	48 hr	72 hr
0	100	100	100
1	93 (6)	101 (5)	103 (4)
10	113 (9)	105 (6)	95 (6)
100	107 (8)	80 (4)*	65 (3)*

Mean (SEM); *$p < 0.05$, $n = 6$.

Table 2. Concentration- and time-dependent effects of water extract of gusuibu (WEGSB) on alkaline phosphatase activity of rat osteoblasts

WEGSB (μg/mL)	Alkaline phosphatase activity (% of control)		
	24 hr	48 hr	72 hr
0	100	100	100
1	105 (7)	98 (6)	97 (4)
10	106 (5)	95 (4)	96 (6)
100	104 (6)	83 (3)*	70 (4)*

Mean (SEM); *$p < 0.05$, $n = 6$.

Table 3. Effects of water extract of gusuibu (WEGSB) and hydrogen peroxide (H_2O_2) on mitochondrial membrane potential of rat osteoblasts

Treatment	Membrane potential (folds of control)
Control	1
WEGSB, 1 μg/mL	0.95 (0.09)
WEGSB, 10 μg/mL	0.92 (0.10)
WEGSB, 100 μg/mL	0.89 (0.12)
H_2O_2, 30 μM	0.92 (0.11)
WEGSB, 1 μg/mL + H_2O_2, 30 μM	0.97 (0.13)
WEGSB, 10 μg/mL + H_2O_2, 30 μM	0.85 (0.19)
WEGSB, 100 μg/mL + H_2O_2, 30 μM	0.65 (0.07)*

Mean (SEM); *$p < 0.05$, $n = 6$.

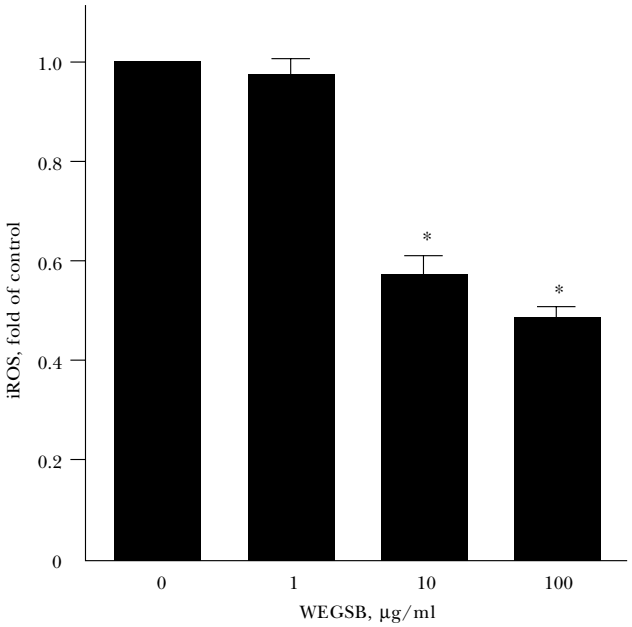

Fig. 1. *Antioxidant effects of water extract of gusuibu (WEGSB) on untreated osteoblasts prepared from neonatal Wistar rat calvarias. Rat osteoblasts were treated with 0, 1, 10, and 100 μg/mL WEGSB for 4 hours. Intracellular reactive oxygen species (iROS) were identified by an ROS-sensitive dye, 2',7'-dichlorofluorescin diacetate, and quantified using a flow cytometer. Each value is represented as mean ± SEM for* n = 6. **Values of WEGSB-treated groups are statistically different from those of the untreated groups as determined by Student's* t*-test,* p < *0.05.*

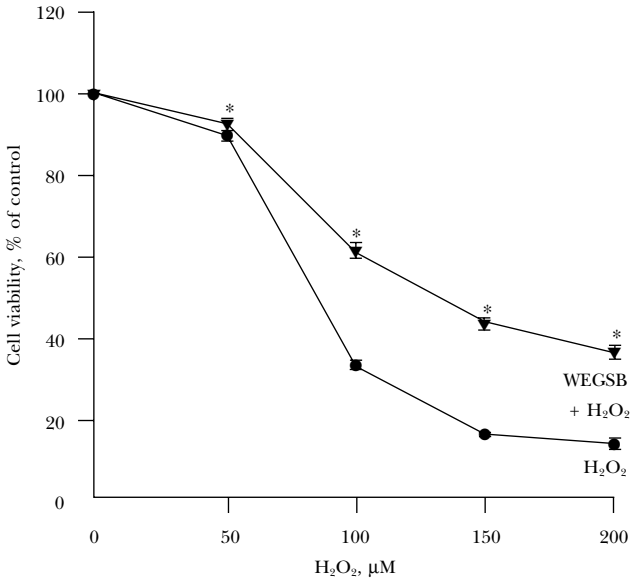

Fig. 2. *Protective effects of water extract of gusuibu (WEGSB) on hydrogen peroxide (H_2O_2)-induced cell death of osteoblasts prepared from neonatal Wistar rat calvarias. Rat osteoblasts were treated with H_2O_2 alone (solid triangle) or a combined treatment of WEGSB (10 μg/mL) and H_2O_2 (solid circle) for 24 hours. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Each value is represented as mean ± SEM for n = 6. *Values for the combination-treated groups were statistically different from those for the respective H_2O_2-treated groups as determined by Student's* t*-test,* p < *0.05.*

inflammation induced by subcutaneous talc or magnesium silicate powder injections causes an inhibition of bone formation and a significant loss of trabecular bone in rats [14, 34]. In an animal model of inflammation-induced osteoporosis, osteoblast numbers and bone mineral densities are reduced [16]. ROS, which are significantly increased by activated immune cells or bone cells during inflammation, can modulate activities of osteoblasts and osteoclasts, and so have been implicated as one of the major causes for inflammation-induced bone defects [13, 16]. Suppression of the levels of ROS may be beneficial to the recovery of bone defects.

Table 4. Effects of water extract of gusuibu (WEGSB; 10 μg/mL) on hydrogen peroxide (H_2O_2)- and nitric oxide (NO)-induced intracellular reactive oxygen species (ROS) of rat osteoblasts

Treatment	Intracellular ROS (folds of control)
Control	1
WEGSB	0.63 (0.04)*
H_2O_2	2.29 (0.31)*
NO	4.64 (0.41)*
WEGSB+ H_2O_2	1.22 (0.19)**
WEGSB+ NO	4.83 (0.50)*

Mean (SEM); *,**Values significantly different from control and H_2O_2-treated group, respectively; $p < 0.05$.

WEGSB has antioxidant effects on rat osteoblasts. In the present study, WEGSB concentration-dependently inhibited the basal levels of intracellular ROS and suppressed hydrogen peroxide-induced intracellular ROS in rat osteoblasts, indicating that WEGSB has antioxidant effects on intrinsic or extrinsic oxidative stress in rat osteoblasts. Flavonoids are a group of chemicals with a diphenylpyran structure and have been shown to have antioxidant properties [35]. The flavonoid-type ingredients extracted from traditional Chinese herbs including *Cynanchum wilfordi*, *Scutellaria baicalensis Georgi*, and ginseng have been found to have antioxidant properties and be able to scavenge free radicals [22, 23, 36]. Naringenin (4',5,7-trihydroxyflavanone) and naringin, a glycoside form of naringenin, have been identified in the extracts of gusuibu [37]. The presence of these two flavonoid

components in WEGSB may explain the antioxidant characteristics of the extract. Therefore, from the activity of antioxidation, WEGSB can suppress oxidative stress in *in vitro* studies and may thus be helpful to bone healing.

The concentration of WEGSB used to treat rat osteoblasts is a critical factor in determining the safety of the extract. Treatment of rat osteoblasts with low concentrations of WEGSB (≤ 10 μg/mL) had no effect on cell viability and alkaline phosphatase activity, a marker for osteoblast metabolism. However, when the concentration of WEGSB reached 100 μg/mL, the extract caused osteoblastic death and decreased alkaline phosphatase activity. Thus, WEGSB at low concentrations (≤ 10 μg/mL) was safe to rat osteoblasts but was toxic at a high concentration (100 μg/mL).

Hydrogen peroxide is an ROS that can increase cellular oxidative stress and adversely affect the physiology and pathophysiology of cells [38]. The present study found that hydrogen peroxide caused a concentration-dependent decrease in osteoblast viability. Combined treatment with WEGSB and hydrogen peroxide significantly inhibited hydrogen peroxide-stimulated osteoblast death. During inflammation, large amounts of ROS, including hydrogen peroxide, are produced. Previous studies have shown that flavonoids can directly scavenge these free radicals [35, 39]. WEGSB is known to contain the flavonoid ingredients naringin and naringenin [37], so the extract may be able to scavenge hydrogen peroxide directly, which then reduces hydrogen peroxide-induced oxidative stress and attenuates hydrogen peroxide-induced signal transduction for cell death or apoptosis. Osteoblasts mediate bone formation and contribute to bone remodeling [10]. Protective effects of WEGSB on osteoblasts from hydrogen peroxide insults may be beneficial to bone recovery.

Combined treatment with WEGSB at a high concentration (100 μg/mL) and hydrogen peroxide disturbed the membrane potential of osteoblastic mitochondria and led to cell death. Disturbing the mitochondrial membrane might damage the energy-generating system resulting in cell dysfunction. *Salvia miltiorrhiza*, a traditional Chinese herb, has been shown to reduce mitochondrial membrane potential and increase cell death in HepG-2 cells [40]. Although the mitochondrial membrane potential of rat osteoblasts was not affected by a single treatment of hydrogen peroxide or WEGSB at 100 μg/mL, combined treatment with WEGSB and hydrogen peroxide significantly reduced the membrane potential. WEGSB is a crude extract with various ingredients, such as, diploptene, naringin and glucose, but which specific component(s) of WEGSB produces the synergistic effect with hydrogen peroxide on the inhibition of mitochondrial membrane potential in rat osteoblasts has not been determined. In the present study, combined treatment with WEGSB and hydrogen peroxide significantly increased osteoblastic death (data not shown). Hortelano et al showed that the alternation of mitochondrial membrane potential caused the release of cytochrome *c* and led to cell death [41].

In conclusion, the present study has demonstrated that WEGSB is not cytotoxic to rat osteoblasts at a concentration of 10 μg/mL or less, but causes osteoblastic death at 100 μg/mL. In the 10μg/mL concentration, WEGSB had antioxidant effects on untreated rat osteoblasts and could protect cells from hydrogen peroxide insults. Further study of the effects of WEGSB on animal models or in clinical patients who are suffering bone defects is needed to determine whether it produces beneficial effects.

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