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Antiosteoporotic activity of 'Dae-Bo-Won-Chun' in the ovariectomized rats

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Running Title : Antiosteoporotic activity and Dae-Bo-Won-Chun

SUMMARY

The preventive effect of herbal formulation, 'Dae-Bo-Won-Chun'(DBWC), on the progress of bone loss induced by ovariectomy (OVX) was studied in rats. From light microseopic analyses, a porous or erosive appearances were observed on the surface of trabecular bone of tibia in ovariectomized rats. Whereas those of the same bone in sham rats were composed of fine particles. The trabecular bone area and trabecular thickness in ovariectomized rats decreased by 50% from those in sham-operated rats, these decreases were completely inhibited by administration of DBWC at concentration of 10mg/kg per day for 7 weeks. The mechanical strength in femur neck was decreased by ovariectomy, and this was significantly suppressed by the administration of DBWC. Serum phosphorus, alkaline phosphatase, and thyroxine levels in ovariectomized rats were increased compared to those in sham-operated rats, and increases were completely inhibited by the administration of DBWC. These results strongly suggest that DBWC is effective in preventing the development of bone loss induced by ovariectomy in rats.

Keywords: Ovariectomy; DBWC; Serum biochemistry; Bone histomorphometry; Mechanical strength

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INTRODUCTION

Osteoporosis which is the main causal factor of bone fractures in elderly persons occurs because of an imbalance between bone formation and bone resorption. This disorder has been increasing remarkably in frequency along with the increase in life expectancy (Riggs and Melton, 1986). Several medications have been reported to be effective for curing osteoporosis based upon the results obtained using an ovariectomized rat models. Estrogen (Ettinger *et al.*, 1987; Kalu, 1991), bisphosphonates (Heaney and Saville, 1976; Kalu, 1991), calcitonin (Mazzuoli *et al.*, 1986; Kalu, 1991), calcium products (Kalu, 1991; Morris *et al.*, 1991), ipriflavone (Agnusdei *et al.*, 1992), and anabolic steroids (Chesnut *et al.*, 1983) are clinically employed as effective medications.

Traditional oriental herbal medicines have been reevaluated by clinicians (Terasawa *et al.*, 1993), because these medicines have fewer side effects and more suitable for long-term use as compared to the chemically synthesized medicines (Xiu, 1988). It has been suggested that the effectiveness of Boerhaavia repens L on low back pain seems to correspond to their efficacy in curing osteoporosis (Koyama *et al.*, 1991). From ancient times, Chinese, Korean, and Japanese women who have had low back pain in climacteric and senescent periods have been treated with traditional oriental medicines. As part of our continuing search for biologically active antiosteoporotic agents from the medicinal resources, DBWC was investigated. DBWC, a traditional oriental herbal medicinal prescription, has been successfully used for the management of osteoporotic disorders in Korea. However, it is still unclear how DBWC prevents osteoporotic disorders in experimental animal models. In the present study, we showed that DBWC prevented the progression of bone loss induced by ovariectomy in rats.

MATERIALS AND METHODS

Preparation of DBWC.

An extract of DBWC was prepared by dissolving natural substances with distilled water. The duration of dissolving was about 30 min. The extract was filtered and then stored at 4℃ before use. The ingredients of 56.25 g DBWC include 7.5 g RADIX GINSENG, *Panax schinseng NESS*, 7.5 g RHIZOMA DIOSCOREAE, *Dioscorea batatas DECAISNE*, 11.25 g RHIZOMA REHMANNIAE, *Rehmannia glutinosa LIBOCH*, 7.5 g CORTEX EUCOMMIAE, *Eucommia ulmoides OLIVER*, 11.25 g RADIX ANGELICAE GIGANTIS, *Angelica gigas NAKAI*, 3.75 g FRUCTUS CORNI, *Cornus officinalis SIEB. et Zucc*, 11.25 g FRUCTUS LYCII, *Lycium chinense MILLER*, and 7.5 g RADIX GIYCYRRHIZAE, *Glycyrrhiza uralensis FISCH.*

These materials were obtained from Oriental Medicine Hospital, Wonkwang University (Iksan, South Korea) and identified by J. Kim, School of Oriental Medicine, Wonkwang University. A voucher specimen (No. 98-01-0008) was deposited at the Herbarium at the College of Pharmacy, Wonkwang University. The yield (w/w) of aqueous form from starting crude materials was about 10%.

Experiment on animals.

Thirty female Sprague-Dawley rats weighing 200-300 g were purchased from Dae Han Experimental Animal Center (Eumsung, Chungbuk, Korea), and the animals were maintained under constant temperature (25 ± 2℃) and humidity (55% ± 5%), and under 12 h/12 h light-dark cycles. The rats were housed individually in standard cages and provided with a commercial standard diet containing 1.2% calcium and 0.8% phosphorous.

At 15 weeks of age, animals were either OVX or Sham-operated (Sham). Bilateral ovariectomies were performed under anesthesia with sodium nembutal administered intraperitoneally. Sham operations were performed by exteriorizing the ovaries. OVX rats were divided into two groups based on treatment with either DBWC or physiological saline. After 7 weeks of treatment, blood was collected and stored at -70℃ until biochemical determinations (see below). The subsequent day the rats were sacrificed. Both femora and tibia were dissected. The right femurs were cleaned from adherent muscles, placed in sterile saline, and stored for 8-16 h at 4℃. Left femurs of 12 and 8 randomly selected animals per group were fixed with Burkhart fixative and used further for histomorphometrical analysis (see below). All procedures using animals were carried out in accordance with the guidelines presented in the Guidline

Principles for the Care and Use of Animals in the Field of Physiological Sciences, published by the Physiological Society of Korea, and were approved by the National Institute of Fitness and Sports Animal Care Committee.

Biochemical analysis.

Serum calcium, phosphate, and alkaline phosphatase were determined by standard laboratory techniques. Serum free T_4 (FT_4) and free T_3 (FT_3) were measured by the Liso-Phase kits (Technogenetics, Milan, Italy) after chromatographic separation of the hormone by Sephadex LH-20 chromatography.

HPLC analysis.

The chromatographic system consisted of a pump (Waters Assoc. 600 HPLC pump), a UV detector (Waters Assoc. 486 tunable absorbance detector), an autosampler (Waters Assoc. 717 plus autosampler) and a data modular (Waters Assoc. 746 data modular). A symmetry C18 column (3.9 × 159 mm, Waters Assoc. USA) was used. 0.05 M NH4H2PO4-Acetonitrile (95:5) was used as the mobile phase. Detection of the peaks at 280 nm and the sensitivity was set at 0.50 AUFS. The injection volume was 10 μl and flow rate was 1.0 mL/min.

Bone histomorphometry.

The left femurs were fixed for 24 h with Brkhardt fixative, cut sagittally into 2 equal halves with a diamond saw (Buehler Isomet, low speed saw, Chicago, IL, USA), dehydrated with methanol and embedded in methylmetacrylate. These longitudinal sections of the proximal femur were cut with an AO Autocut/Jung 1150 microtome at 4 μm thickness. The sections with the widest marrow cavity near the central part of the femoral neck were selected for further histological processing and histomorphometric measurements. The 4 m thick sections were stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA) for measurements of cancellous bone volume, osteoblast surface, and osteoclast surface.

All bone measurements were performed with the Bioquant Bone Morphometry System (R & M Biometrics Corp. Nashville, TN) as previously described (Wronski *et al.*, 1993). Cancellous bone measurements were performed in the proximal femur in an area begining 1 mm distal to the growth plate-metaphyseal junction and extending further distally to the junction of the femoral neck and greater trochanter.

Cancellous bone volume as a percentage of bone tissue area and osteoblast and osteoclast surfaces as percentages of total cancellous perimeter were measured at a magnification of × 200. Trabecular number, width, and separation were calculated.

Measurement of mechanical strength of the femur neck.

The retrieved femurs were harvested and embedded in rectangular epoxy blocks, fitting the grips of the biomechanical testing using universal testing machine (Instron-4467).

Statistical analysis.

A software computer program (SAS, SAS Institute Inc. Cary, NC, USA) was used. Intergroup differences were analyzed by one-way analysis of variance (ANOVA), and Tukey's studentized Range test was used to compare pairs of means. These parametric statistical tests could be used because the data were normally distributed. Bone histomorphometry results were analyzed by nonparametric statistics using ANOVA and Wilcoxon tests.

RESULTS

Effect of DBWC on body weight

The body weight of ovariectomized (OVX) rats was significantly higher than that of sham control (sham) rats. After 7 weeks, the body weights in sham and OVX rats were 264 ± 21 g (mean ± S.D., n=10) and 304 ± 27 g (n=10), respectively. Increases in the body weight of animals treated with DBWC (OVX+DBWC) were almost the same as those in OVX rats (Fig.1).

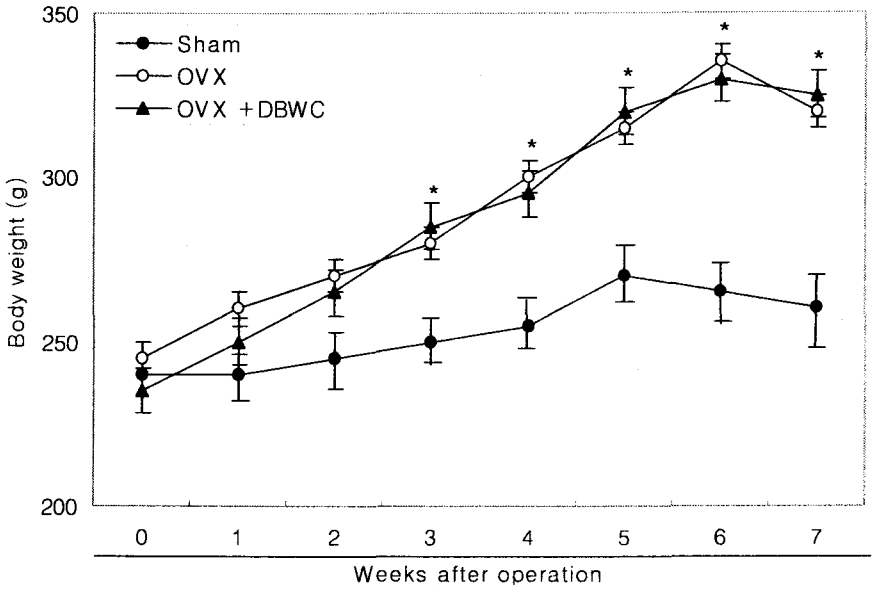

Fig 1. Changes in body weight in rats. Rats were Sham-operated group (Sham: •), ovariectomized group (OVX: •), and OVX + DBWC group (•). Each point represents the mean value ± SD (n=10). *: Statistical significance as compared with Sham group (*: $p < 0.05$).

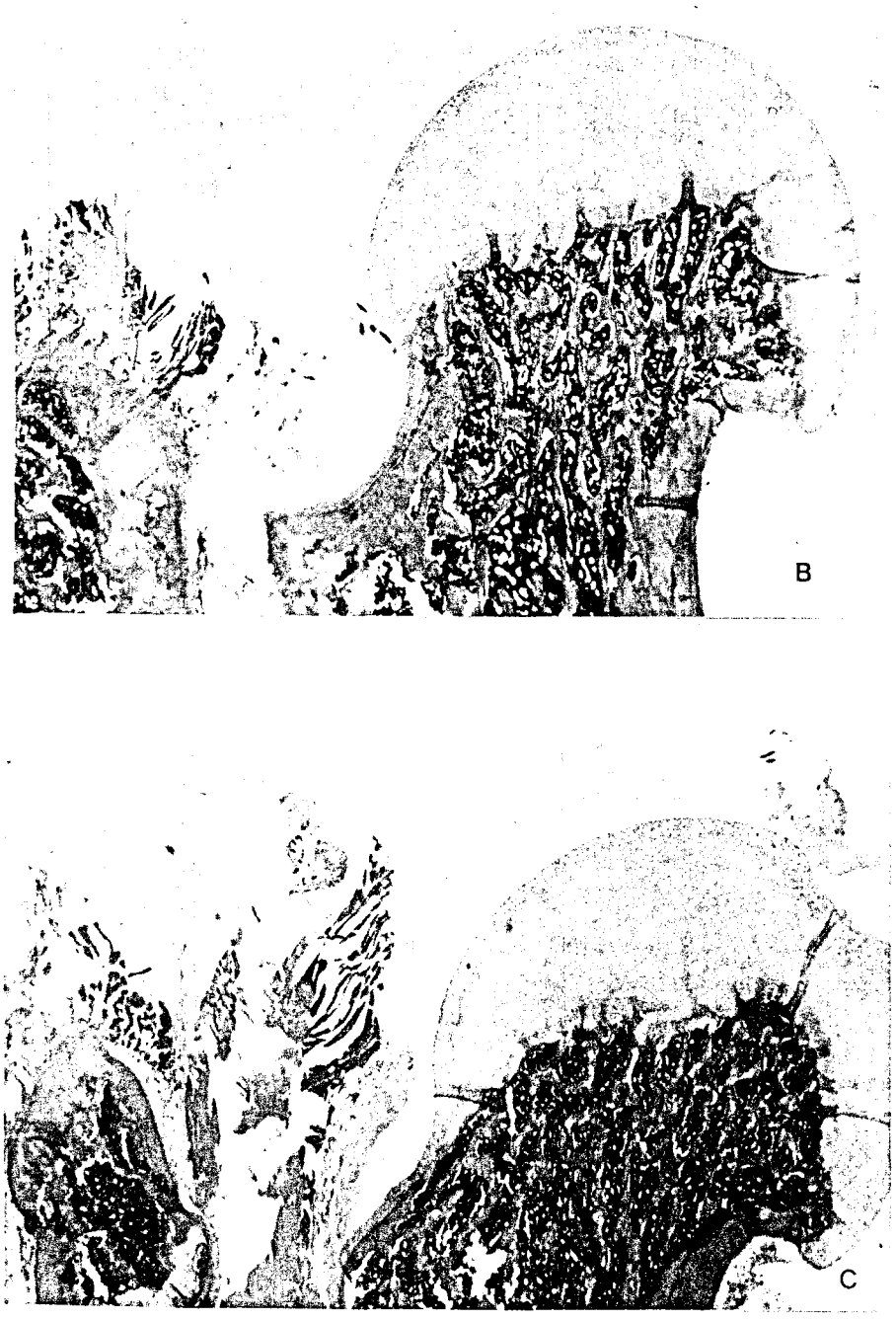

Fig 2. The sample area within the femoral neck for measurement of trabecular bone variables is depicted by the black lines. It begins 1 mm distal to the growth plate (GP) and extends nearly to the junction of the femoral neck and greater trochanter (GT).

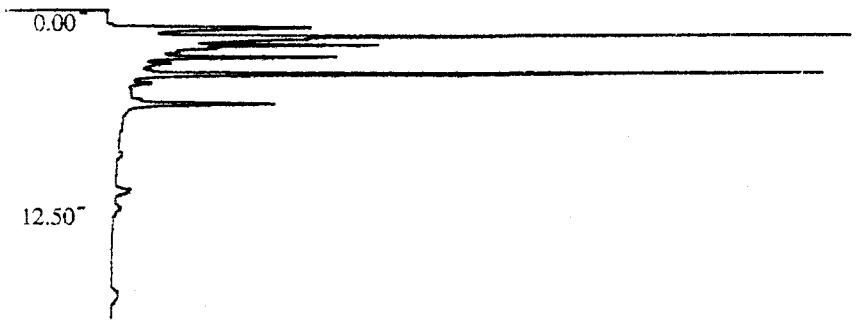

Fig 3. HPLC chromatogram of the DBWC. Standard solution of DBWC was prepared by dissolving in distilled water (1 mg/ml). The solution was filtered through 0.45 μm membrane filter and applied to HPLC. The injection volume was 10 μl and the detection was made at 280 nm with the detector range of 0.50 AUFS.

Effect of DBWC on the bone histology

At 7 weeks after operation, the trabecular bone area in OVX was significantly lesser than the value of sham. A decrease in trabecular bone area induced by ovariectomy was inhibited by the administration of DBWC for 7 weeks (Table 1).

Table 1. Effect of DBWC on the trabecular bone area (%) of the ovariectomized rats.

Groups	No. of animals	Trabecular bone area (%)
Sham	10	56.95±8.2
OVX	10	33.43±5.1***
OVX+DBWC	10	41.46±4.51*

Values are mean ± standard deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (***: $p < 0.001$). *: Statistical significance as compared with OVX group (*: $p < 0.05$)

At 7 weeks after operation, the trabecular thickness in OVX was significantly lesser than the value of sham. A decrease in trabecular thickness induced by ovariectomy was inhibited by the administration of DBWC for 7 weeks (Table 2).

Table 2. Effect of DBWC on the trabecular thickness (m) of the ovariectomized rats.

Groups	No. of animals	Trabecular thickness (m)
Sham	10	95.24±8.8
OVX	10	46.92±5.1***
OVX+DBWC	10	76.14±13.56***

Values are mean ± standard deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (***: $p < 0.001$). #: Statistical significance as compared with OVX group (***: $p < 0.001$).

The trabecular number in OVX was lesser than the value of sham. A decrease in trabecular number induced by ovariectomy didn't inhibit by the administration of DBWC for 7 weeks (Table 3).

Table 3. Effect of DBWC on the trabecular number (No/mm) of the ovariectomized rats.

Groups	No. of animals	Trabecular number (No/mm)
Sham	10	5.44±0.5
OVX	10	4.74±1.1
OVX+DBWC	10	4.82±0.79

Values are mean ± standard deviation. OVX: Ovariectomized.

The trabecular separation in OVX was greater than the value of sham. An increase in trabecular separation induced by ovariectomy was inhibited by the administration of DBWC for 7 weeks (Table 4).

Table 4. Effect of DBWC on the trabecular separation (μm) of the ovariectomized rats.

Groups	No. of animals	Trabecular separation (μm)
Sham	10	109.07±23.4
OVX	10	147.98±41.1
OVX+DBWC	10	103.85±10.32*

Values are mean ± standard deviation. OVX: Ovariectomized. *: Statistical significance as compared with OVX group (*: $p < 0.05$).

The osteoclast number and osteoblast surface in OVX were significantly greater than the respective values of sham. The increase of osteoclast number and osteoblast surface induced by ovariectomy was inhibited by the administration of DBWC for 7 weeks (Table 5).

Table 5. Effect of DBWC on the osteoclast number (No/mm) and osteoblast surface (%) of the ovariectomized rats.

Groups	No. of animals	Osteoclast number (No/mm)	Osteoblast surface (%)
Sham	10	0.58±0.2	7.20±1.6
OVX	10	1.67±0.6***	21.94±6.7***
OVX+DBWC	10	1.63±0.53	19.27±3.91

Values are mean ± standard deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (***: $p < 0.001$).

Effect of DBWC on the mechanical strength of the femur neck of the ovariectomized rats

The mean mechanical strength of femur neck was 33.86 ± 4.25 (N) in sham group, 25.39 ± 5.89 (N) in OVX group, and 29.73 ± 6.49 (N) in OVX + DBWC group. The mechanical strength in femur neck was decreased by ovariectomy, and this was significantly suppressed by the treatment of DBWC for 7 weeks (Table 6).

Table 6. Effect of DBWC treatment on the mechanical strength of the femur neck of the ovariectomized rats.

Groups	No. of animals	Mechanical strength (N)
Sham	10	33.86±4.25
OVX	10	25.39±5.89*
OVX+DBWC	10	29.73±6.49

Values are mean ± standard deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (*: $p < 0.05$).

Effect of DBWC on serum biochemical levels

To assess the contribution of DBWC to factors involved in antiosteoporotic reactions, we conducted the serum biochemical analysis. At 7 weeks after ovariectomy, serum calcium level was not changed significantly by ovariectomy, but phosphorus, alkaline phosphatase, and thyroxine levels in OVX were significantly higher than those in Sham. However, serum alkaline phosphatase and thyroxine levels in OVX were decreased by treatment of DBWC for 7 weeks. Also, the level of serum calcium in OVX was suppressed by 7 weeks DBWC treatment (Table 7).

Table 7. Effect of DBWC treatment on serum biochemical levels.

Groups	No. of animals	Calcium (mg/dl)	Phosphorus (mg/dl)
Sham	10	10.97±0.37	5.60±0.88
OVX	10	10.80±0.70	7.31±1.09**
OVX+DBWC	10	10.10±0.51*	7.55±1.91

Alkaline phosphatase (IU/L)	Triiodothyronine (T3; ng/ml)	Thyroxine (T_4; g/dl)
122.50±29.24	1.10±0.19	1.02±0.31
324.60±43.05**	1.16±0.11	3.23±0.56***
226.00±65.70*	1.23±0.41	2.21±4.91**

Values are mean ± standard deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (**: $p < 0.01$; ***: $p < 0.001$). *: Statistical significance as compared with OVX group (*: $p < 0.05$; **: $p < 0.01$)

DISCUSSION

Osteoporosis, which has been defined as a "state of low bone mass", is one of the major problems in our aging society. Osteoporosis results in bone fracture in older members of the population, especially in postmenopausal women (McGowan, 1993). In traditional medicine, there are many natural crude drugs that have the potential for use to treat bone diseases; however, not much laboratory work has been reported evaluating this possible use. In a search for natural crude drugs having inhibitory activity on bone resorption, we have screened a number of plants widely used in traditional medicine for their inhibitory activity on bone resorption induced by ovariectomy.

Ovariectomy caused an increase in body weight (Fig. 1). This is one of the prominent features that have been postulated to provide a partial protection against the development of osteoporosis in long bones (Roudebush *et al.*, 1993). It has been reported that both bone resorption and bone formation are promoted by ovariectomy and the prominent increase of the bone resorption is termed high- turnover osteoporosis (Wronski *et al.*, 1988). It is possible that the porous appearance of the femoral head in ovariectomized rats resulted from this high-turnover osteoporosis.

In animals and humans, loss of ovarian function causes dramatic changes in bone mass, due to an imbalance between the amount of resorbed bone and that formed at each remodeling site. Internal microarchitecture and strength are also impaired, leading to increased bone fragility, especially in metaphyseal regions (Melton *et al.*, 1988). Nowadays, an ovariectomized animal is commonly used as a postmenopausal osteoporosis model.

In this study, the ovariectomized rat induced a decrease in serum phosphorus level but calcium level does not change. Alkaline phosphatase is the most widely recognized biochemical marker for osteoblastic activity. Although its precise mechanism of action is poorly understood, this enzyme is believed to play a role in bone mineralization. The ovariectomized rat induced a significant increase in serum alkaline phosphatase.

Thyroid hormones play an important role in bone remodeling (Mosekilde *et al.*, 1990), and histomorphometric studies have shown that thyroid hormones stimulate osteoblastic and osteoclastic activities in cortical and trabecular bone (Mosekilde *et al.*, 1977). Thyrotoxicosis is associated with increased bone turnover, and the resorption rate exceeds the formation rate, thus resulting in bone loss (Wartofsky, 1988). The ovariectomized rat induced a significant increase in serum thyroxine (T_4) levels.

The present study showed that DBWC treatment profoundly affected ovariectomized-induced serum biochemical change. DBWC inhibited serum calcium level, serum alkaline phosphatase activity, and serum thyroxine level compared to those in ovariectomized rats. Also, DBWC increased the mechanical strength of femur neck (29.73 ± 6.49 N) compared to that in ovariectomized rats (25.39 ± 5.89 N). Next, we investigated that the effects of DBWC on trabecular bone volume were studied. Trabecular bone volume was measured 4 μm decalcified and stained thin bone slice by image analysis using a digitalizer. DBWC inhibited the decrease in trabecular bone area (%), trabecular thickness (μm), and trabecular number (No/mm) induced by ovariectomy and also the increase in trabecular separation (μm) induced by ovariectomy.

In conclusion, the results obtained in the present study provide evidence that DBWC importantly contributes to preventing or treating the development of bone loss induced by ovariectomy in rats. The studies on the isolation and characterization of the active chemical constituents are in progress.

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