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Turbo methanol extract inhibits bone resorption through regulation of T cell function

Babita Balakrishnan [a], Madhavi M. Indap [a], Surya P. Singh [b], C. Murali Krishna [b], Shubhada V. Chiplunkar [c,*]

[a] Department of Zoology, The D. G. Ruparel College, Mahim, Mumbai 40016, India
[b] Chilakapati Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai 410210, India
[c] Chiplunkar Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai 410210, India

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ABSTRACT

Marine organisms have bioactive potential which has tremendous pharmaceutical promise. Emerging evidence highlights the importance of the interplay between bone and the immune system of which T lymphocytes and their product act as key regulators of bone resorption. In the present investigation we have analyzed the anti-osteoporotic effect of turbo methanol extract (TME) in the reversal of bone resoprtion. Forty-two female Swiss albino mice were used and randomly assigned into sham-operated group (sham) and six ovariectomized (OVX) subgroups, *i.e.* OVX with vehicle (OVX) that received daily oral administration of water *ad libitum*; OVX with estradiol (2 mg/kg/day); and OVX with different doses of TME *i.e.* TME 100 mg/kg, TME 50 mg/kg, TME 25 mg/kg and TME 12.5 mg/kg. Oral administration of TME or estradiol started on the second week after ovariectomy for a period of 4 weeks. We observed that the administration of TME increased the trabeculation in tibia and reduced the atrophy in the uterus. TME significantly decreased the serum alkaline phosphatase (ALP) and acid phosphatase (ACP) activity in OVX mice. Micro CT analysis revealed that the TME administration preserved the bone volume, connectivity density, trabecular number, trabecular thickness and trabecular separation in OVX mice. Bone mineralization was measured in different groups of mice by Raman spectroscopy. Reversal of bone resorption was observed in TME treated group of mice. To further investigate the mechanism of action of TME, we analyzed the T lymphocyte proliferation and profiles of cytokine TNFα and sRANKL in TME treated ovariectomized mice. Decrease in the elevation of T cell subsets was observed after the supplementation with TME. The extract significantly lowered the T cell proliferation responses to mitogens, phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) and phytohemagglutinin (PHA). A marked reduction in TNFα and sRANKL secretion in serum and TNFα in cell free supernatants of activated T lymphocytes was observed upon TME administration. TME could significantly inhibit the *in vitro* osteoclastogenesis and the bone resorption observed using artificial calcium coated slides. Collectively, these results indicate that TME has the potential to inhibit bone resorption and may prove to be a potential candidate for the development of an anti-osteoporosis drug.

Introduction

Osteoporosis characterized by the loss of bone mass and strength that leads to fragility fractures has probably existed throughout human history but only recently became a major clinical dilemma as human life span increased. Postmenopausal osteoporosis has become a foremost problem with significant morbidity and mortality. After the onset of menopause, the cessation of the estrogen leads to increased bone turnover with an increase in bone resorption by osteoclasts, resulting in decreased bone mass [1]. This progressive bone loss caused by the sharp decrease in ovarian estrogen production can be prevented by estrogen replacement therapy (ERT) [2]. However, estrogen therapy recently became a subject of debate because clinical studies revealed an increased risk of breast cancer and coronary artery disease in women who take estrogen supplement [3]. Thus it is necessary to develop naturally occurring compounds with less undesirable side effects that can substitute or reduce the need for the drugs used currently.

Estrogen deficiency induced osteoporosis leads to a marked stimulation of bone resorption which is caused primarily by increased osteoclast (OC) formation. Osteoclast formation is regulated by the production of RANKL (receptor activator of nuclear factor kappa-B ligand) and M-CSF (macrophage-colony stimulating factor) under the influence of stimulators of resorption leading to the maturation and activation of the osteoclast so formed to resorb bone. RANKL exists in two forms, soluble and membrane bound. Soluble form is secreted by T lymphocytes and membrane bound RANKL is expressed by the

Abbreviations: TME, turbo methanol extract; OVX, ovariectomized mice; ALP, alkaline phosphatase; ACP, acid phosphatase; TNFα, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; Io, ionomycin; PHA, phytohemagglutinin; RANKL, receptor activator of nuclear factor kappa-B ligand; M-CSF, macrophage-colony stimulating factor; sRANKL, soluble RANKL; OC, osteoclast; ERT, estrogen replacement therapy; α-MEM, α-minimum essential medium; FBS, fetal bovine serum.

* Corresponding author. Fax: +91 22 27405085.
E-mail address: schiplunkar@actrec.gov.in (S.V. Chiplunkar).

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osteoblastic lineage cells. Overexpression of soluble RANKL (sRANKL) in transgenic mice leads to skeletal deformities which are similar to postmenopausal osteoporosis [4]. The T cell expression of RANKL can be seen *in vivo* at sites of increased bone resorption thus proving the role of T cells in augmenting bone resorption [5]. Among the factors that upregulate osteoclast formation and lead to bone loss in estroprevic humans and rodents is tumor necrosis factor α (TNFα) [6]. TNFα stimulates OC activity and inhibits osteoblastogenesis thus further driving an imbalance between bone formation and bone resorption [7]. Studies in mice suggest that activated T cells are the most relevant source of TNFα in conditions of estrogen deficiency [6–8]. T cells are key inducers of bone wasting because ovariectomy increases T cell TNFα production to a level sufficient to augment RANKL-induced osteoclastogenesis. Some studies have attested to the relevance that ovariectomy fails to induce bone loss in mice lacking TNFα or its type I receptor [7]. Athymic mice (*nu*/*nu*) lacking T cells, do not exhibit bone loss after ovariectomy, in contrast to their control littermates (nu/+) [9]. Immune cells can regulate bone cell activities and the effects of estrogen on immune cells are more important for the bone loss sparing effect [10].

Marine organisms harbor surplus structurally unique and biologically active secondary metabolites that have enormous potential for treatment of variety of diseases [11]. Targeted strategies in combination with high throughput screening are being employed in this hunt for novel pharmacotherapeutic agents. In the search for active components from marine organisms colonial zoanthid *Zoanthus* sp. have proved to be a good candidate as an anti-osteoporosis drug [12]. *Turbo brunneus* (Röding) used in this study belongs to the phylum Mollusc, family Turbinidae found exclusively in the rocky shores in mesolittoral zone [13]. There is lack of scientific data regarding the anti-osteoprotective activity of the mollusc *T. brunneus* which prompted us to undertake the present study. In this study, we show that the methanol extract of *T. brunneus* was able to inhibit the bone resorption *in vivo* and *in vitro*. This extract was also able to inhibit the estrogen deficiency induced increase in T cell proliferation, production of osteoclastogenic cytokine, TNFα and also bone resorption.

Materials and methods

Preparation of TME

The organism *T. brunneus*, exposed during low-tide was collected from the rocky shore of Ratnagiri, Maharashtra, India. It was deshelled and the body mass (700 g) was cold percolated in methanol for 3–4 days. The resulting methanol extract was filtered and the residual animal mass was again immersed in fresh methanol. The process was repeated until the methanol extract became colorless. The filtered extracts were pooled and concentrated under reduced pressure and crude gummy methanol extract was obtained with a yield of 1.82%. It was refrigerated at −20 °C until further use.

Animals and treatments

Female Swiss albino mice of 18–22 g maintained at the Animal House of ACTREC were used for the study. The study was approved by Institutional Animal Ethics Committee, ACTREC. They were fed with standard laboratory diet and water *ad libitum*. The mice were randomly divided into 7 groups, consisting of 4 animals in each group.

Group A Bilaterally ovariectomized control mice treated with vehicle (OVX)
Group B Sham control mice treated with vehicle (sham control)
Group C Bilaterally ovariectomized control mice treated with estradiol-2 mg/kg body weight (positive control)
Group D Bilaterally ovariectomized control mice treated with TME-12.5 mg/kg body weight
Group E Bilaterally ovariectomized control mice treated with TME-25 mg/kg body weight
Group F Bilaterally ovariectomized control mice treated with TME-50 mg/kg body weight
Group G Bilaterally ovariectomized control mice treated with TME-100 mg/kg body weight

All mice were bilaterally ovariectomized after anesthetizing with ketamine + xylazine. After 7 days of recovery from surgical convalescence, animals of Groups D, E, F and G were administered orally through stomach tube with TME 12.5 mg/kg body weight, 25 mg/kg body weight, 50 mg/kg body weight, 100 mg/kg body weight of TME respectively.

The vehicle (sterilized distilled water) or TME dissolved in sterilized water was administered daily for a period of 4 weeks. Animals in the control Groups A and B received water only (vehicle control) and of those in Group C received estradiol (positive control). The body weight of the animals was recorded weekly during the experimental period.

The ovariectomized mice or rat models mimic changes in bone metabolism observed in postmenopausal osteoporosis and have been the most commonly used for the mechanistic and efficacy studies of potential therapeutic agents for the treatment and prevention of osteoporosis [14].

Body weight measurements

On completion of the experimental period, the final body weights of animals of all the 7 groups (Groups A to G) were recorded. The animals were sacrificed under deep anesthesia on the scheduled date and weights of uteri were recorded.

Histopathological examination

Tissue collection and processing. The tibia and uterus were harvested rapidly and fixed with Bouin's solution for 2 h, which was followed by decalcification in 5% nitric acid for tibia, dehydration in alcohol, clearing in xylene & finally embedding in paraffin for further tissue section and histological staining [15].

Bone microarchitecture analysis by micro-CT analysis

The proximal tibia of each group of mice was scanned with a high resolution by micro-CT 40 system (Scanco Medical, Switzerland). The parameters for each single scan were 70 kVp of the X-rays and with intensity of 114 μA. The scan distance and the sample time for each sample were 12,288 μm and 300,000 μs respectively. The increment and thickness between the slices were 12 μm each. Morphologic measurements of the proximal trabecular bone were performed and the following 3D parameters were obtained: bone volume (BV)/tissue volume (TV), (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and connectivity index.

Raman spectroscopic examination of control and TME treated bones

Raman spectra were recorded from the shaft region of the bones obtained from OVX control, sham control, estradiol and TME (100, 50, 25, 12.5 mg/kg) treated groups of mice as described earlier. The spectra were recorded at different points with spacing of 1 mm and were acquired using HE-785 Raman spectrometer (LabRam, Jobin-Vyon-Horiba, France). Briefly, this system consists of a diode laser of 785 nm wavelength as excitation source and a high efficiency (HE-785) spectrograph coupled with a CCD (Synapse) as detection element. Optical filtering of unwanted noise including Rayleigh signals is accomplished through 'Superhead' component of the system. Superhead coupled with a 100× microscopic objective (Nikon, NA 0.65) was used to deliver laser light as well as to collect Raman signals. The spectral resolution as per manufacturer's specification is approximately 4 cm^{-1}. Estimated laser spot size at the sample was 2–3 μm. Spectra were integrated for 5 s and averaged over 10 accumulations. Laser power at