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Effects of tocotrienol from *Bixa orellana* (annatto) on bone histomorphometry in a male osteoporosis model induced by buserelin

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ABSTRACT

Introduction: Osteoporosis is a debilitating skeletal side effect of androgen deprivation therapy based on gonadotropin-releasing hormone (GnRH) agonist in men. Tocotrienol from *Bixa orellana* (annatto) has been demonstrated to offer protection against osteoporosis by exerting anabolic effects on bone. Thus, it may prevent osteoporosis among GnRH agonist users.

Objective: This study aimed to determine the effectiveness of annatto-tocotrienol on the bone turnover markers and bone histomorphometry in a model of male osteoporosis induced by buserelin (a GnRH agonist).

Methods: Forty-six three-months-old male Sprague-Dawley rats (three months old; 300–350 g) were randomly divided into six groups. The baseline control group (n = 6) was sacrificed at the onset of the study. The normal control group (n = 8) received corn oil (the vehicle of tocotrienol) orally daily and normal saline (the vehicle of buserelin) subcutaneously daily. The buserelin control (n = 8) received corn oil orally daily and subcutaneous buserelin injection 75 μg/kg/day daily. The calcium control (n = 8) received 1% calcium in drinking water and subcutaneous buserelin injection 75 μg/kg/day. The remaining rats were treated with two different treatments, i.e., (1) oral annatto tocotrienol at 60 mg/kg/day plus subcutaneous buserelin injection 75 μg/kg/day (n = 8); (2) oral annatto tocotrienol at 100 mg/kg/day plus subcutaneous buserelin injection 75 μg/kg/day (n = 8). The rats were injected with calcein twice before being sacrificed to label the bones. The rats were euthanized, and their blood and right femur were harvested at the end of the treatment for bone turnover markers and bone histomorphometry examination.

Results: Both serum osteocalcin and C-telopeptide of type 1 collagen were not significantly different between treated groups and buserelin control ($P > 0.05$). The buserelin control group had a significantly lower bone volume and higher eroded surface compared with the normal control group ($P < 0.05$). Both groups treated with annatto tocotrienol (60 mg/kg/day and 100 mg/kg/day) had significantly higher bone volume, trabecular thickness and osteoblast number, as well as a significantly lower single-labelled surface compared with the buserelin control ($P < 0.05$). Only rats treated with annatto tocotrienol 60 mg/kg/day had a significantly higher double-labelled surface compared with buserelin control ($P < 0.05$).

Conclusion: Annatto tocotrienol can prevent trabecular bone loss by increasing the mineralising surface and osteoblasts number. Thus, it has a potential role in preventing bone loss in men using GnRH agonist.

1. Introduction

Bone is a highly specialized and rigid connective tissue consisting of inorganic and organic components [1]. It undergoes continuous remodelling governed by specialized bone cells in response to physical and physiological demands of the body. These cells include osteoblasts responsible for bone formation, osteoclasts responsible for bone resorption, as well as osteocytes which act as a mechanosensor in regulating the bone remodelling process [2]. Enzyme and proteins released into the circulation during bone formation and resorption can be used to monitor bone remodelling process during drug treatment. Osteoporosis occurs when the rate of bone resorption is greater than bone formation [3].

Deterioration of skeletal microstructure precipitates bone fragility and fracture. Androgen deficiency is the major cause of male osteoporosis [4]. Androgens affect bone cells by binding to the androgen receptors directly or oestrogen receptors indirectly via aromatization to oestrogen [5]. Activation of androgen receptor promotes the

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differentiation of osteoblasts and bone formation while suppressing the formation of osteoclasts and bone resorption [5]. Male hypogonadism can be divided into primary (due to ageing and testicular failure) and secondary hypogonadism (due to disturbance in hypothalamic–pituitary–gonadal axis). Hormonal agents used in androgen deprivation therapy, like gonadotropin-releasing hormone (GnRH) agonists, can induce secondary hypogonadism due to suppression of gonadotropins secretion [6]. Buserelin is a classic example of GnRH agonist used in the treatment of prostate cancer, precocious puberty and transgender hormone therapy. Human studies showed that both circulating bone formation and resorption markers increase progressively with the use of GnRH agonists, indicating increased bone turnover [7]. Men receiving GnRH agonists also suffered from an accelerated bone loss at a rate of 3–5% annually and increased risk for fragility fracture at vertebral, hip or femur [8,9]. Fragility fracture is a serious health concern because it incurs increased mortality, loss of productivity and high healthcare costs among osteoporotic patients [10]. The current antiosteoporosis agents, such as bisphosphonates, teriparatide and denosumab, are effective in increasing bone mineral density and preventing fracture in patients despite a few side effects. However, the preventive agent for osteoporosis is limited to calcium with or without vitamin D. This prompted the search of a new agent with the potential to prevent bone loss among high-risk individuals.

Tocotrienol is a division of vitamin E family that possesses stronger antioxidant and anti-inflammatory effects than its tocopherol counterpart [11,12]. Previous studies have demonstrated that tocotrienol exerts antiosteoporotic effects in various animal models of osteoporosis [13–16]. Vitamin E derived from annatto (*Bixa orellana*) bean contains a high amount of delta-tocotrienol (90%) and gamma-tocotrienol (10%) but no alpha-tocopherol [17]. The unique composition of annatto vitamin E provides an opportunity to study the bone protective effects of tocotrienol without the interference of alpha-tocopherol. Alpha-tocopherol might reduce the bioavailability and biological activity of tocotrienol [18,19]. A study by Fujita et al. found that high-dose alpha-tocopherol supplementation in mice increased bone resorption due to increased osteoclast formation [20]. However, further studies failed to show that high-dose alpha-tocopherol or palm tocotrienol mixture exerted negative effects on bone in ovariectomy female rats and normal male rats [21–23]. Oral supplementation of annatto tocotrienol (60 mg/kg/day for eight weeks) has been shown to increase osteoblast number and osteoid surface, while reducing osteoclast number and eroded surface in orchidectomized male rats [24]. The skeletal protective effects of tocotrienol on osteoporosis induced by GnRH agonists have not been explored. Some studies suggest that skeletal consequences caused by secondary hypogonadism are more severe compared to primary hypogonadism [25–28].

The purpose of the current study was to determine the effects of annatto tocotrienol on bone remodelling markers (CTX and osteocalcin) and bone histomorphometric parameters in male osteoporosis model induced by buserelin, a GnRH agonist. We hypothesized that annatto tocotrienol could prevent deterioration of bone in male rats receiving buserelin. We hope the findings from this study will validate the use of annatto tocotrienol as a preventive agent against osteoporosis in men receiving GnRH agonist.

2. Materials and methods

2.1. Animals and treatment

Forty-six three-months-old male Sprague-Dawley rats were procured from the Laboratory Animal Resource Unit of Universiti Kebangsaan Malaysia (Kuala Lumpur, Malaysia). The rats were housed individually in plastic cages at the animal Laboratory in the Department of Pharmacology, University Kebangsaan Malaysia (Kuala Lumpur, Malaysia) under standard conditions (27 °C, natural dark-light cycle). They were provided with standard rat chow (Gold Coin Holdings, Kuala Lumpur, Malaysia) containing 0.8% of calcium by weight (based on food label provided by the manufacturer) and tap water containing 6.65 mg/L calcium (based on national average value [29]) ad libitum. After a week of acclimatization, the rats were randomized into six groups. The baseline control (BC) group (n = 6) was sacrificed at the onset of the study. The normal control group (NC) (n = 8) received corn oil (the vehicle of tocotrienol) orally daily and normal saline (the vehicle of buserelin) subcutaneously daily. The buserelin control (BuC) (n = 8) received corn oil orally daily and subcutaneous buserelin injection at 75 µg/kg/day. The calcium control (CaC) (n = 8) was given 1% calcium in drinking water and subcutaneous buserelin injection at 75 µg/kg/day. The remaining rats were treated with two different treatments, i.e., (1) oral annatto tocotrienol at 60 mg/kg/day plus subcutaneous buserelin injection 75 µg/kg/day (n = 8); (2) oral annatto tocotrienol at 100 mg/kg/day plus subcutaneous buserelin injection 75 µg/kg/day (n = 8). The dose of buserelin used was based on a pilot study, which showed that serum testosterone and bone microstructure deteriorated significantly in male rats at this dose after three months [30,31]. The protocol of the experiment was reviewed and approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (Approval Code: FP/FAR/2015/CHIN/29-JULY/698-JULY-2015-MAY-2017).

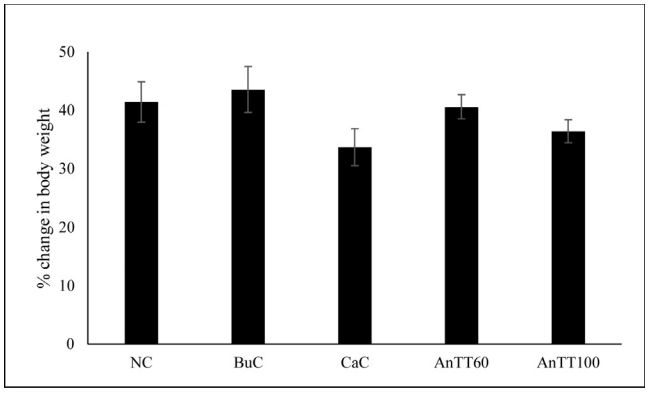

Fig. 1. Percentage changes in body weight after 3 months of treatment. The data are shown as mean ± standard error of the mean.
Abbreviation:NC = normal group; BuC = buserelin group; CaC = calcium group; AnTT 60 = annatto-tocotrienol 60 mg/kg group; AnTT 100 = annatto-tocotrienol 100 mg/kg group.

2.2. Bone turnover markers

The blood of the rats was collected using plain tubes via tail vein at the beginning of the study and cardiac puncture at sacrifice under anaesthesia. It was centrifuged at 3000 rpm for 10 min to extract the serum. It was then stored at −70 °C until analysis. Serum C-telopeptide of type 1 collagen (CTX-1) and osteocalcin level were measured using enzyme-linked immunosorbent assay (Wuhan Fine Biotech Co. Ltd, China).

2.3. Bone histomorphometry

The rats were injected with calcein nine days and two days before sacrifice to label the bones. After three months of treatment, the rats were euthanatized and their right femurs were dissected and fixed with 15% formalin for one week after being sawed into halves. A part of the bone samples was decalcified using ethylenediaminetetraacetic acid (Sigma-Aldrich, Saint Louis, USA) for eight weeks while the other part was not. Undecalcified bone samples were embedded in polymer methyl methacrylate (Osteo-Bedd Bone Embedding Kit; Polysciences, Warrington, PA), and sectioned at 9 µm thickness using a microtome (Leica, Wetzlar, Germany). Some sections were stained using von