



Original Article

Changes of condyle cartilage in orchidectomized rats fed with young coconut juice: Novel preliminary findings

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Received 6 November 2009; Accepted 6 July 2010

Abstract

Androgens play a very important role in building the skeleton in young adults and help to prevent bone loss and osteoporosis in aging men. In addition, in elderly men, bone mass has been related to estrogen levels rather than to testosterone. Estrogen replacement therapy has, therefore, been proposed to prevent bone loss in males as well as in females. Estrogen, however, has been considered as one of the hormonal risk factors of benign prostatic hyperplasia and prostate cancer and also other side effects. Young coconut juice (Y CJ), presumably containing phytoestrogen, was investigated in the present study for its possible beneficial effects on delaying osteoporosis using a male rat model, to replace estrogen replacement therapy. In this study, mandibular condylar cartilage was used as the osteoporotic model. We have found that total cartilage thickness, particularly the hypertrophic zone of mandibular condylar cartilage, was thickest in the sham-operated rats receiving Y CJ orally fed for a 14-day period, compared with a sham, orchidectomized, orchidectomized rats receiving estradiol benzoate, and orchidectomized rats receiving Y CJ.

Keywords: young coconut juice, osteoporosis, bone, orchidectomy, condyle cartilage

1. Introduction

Androgens play a role in building the skeleton of young adults (Albright and Reifenstein, 1948) and also help to prevent bone loss and osteoporosis in aging men. Testosterone, one of androgen forms, is converted into 17 β -estradiol (E2), and increasing evidence indicates that the effect of androgens on bone metabolism is mediated by their aromatization to estrogens (Riggs *et al.*, 2002; Khosla *et al.*, 2002). Estrogens are very important for skeletal maturation and

epiphyseal closures in both men and women, and androgens play significant direct roles in skeletal development. It is not yet clear whether trabecular thickening or periosteal bone formation is directly affected by androgens, or primarily by estrogens aromatized from androgens (Riggs *et al.*, 2002). Men and women with low gonadal sex steroid levels are associated with rapid bone loss (Hofbauer *et al.*, 2002).

In rats and mice, ovariectomy (ovx) induced estrogen deficiency and this affected temporomandibular joint (TMJ) development (Okuda *et al.*, 1996; Yamashiro and Takano-Yamamoto, 1998; Yasuoka *et al.*, 2000; Cheng *et al.*, 2000; Fujita *et al.*, 1998, 2001). Ovx caused significant changes, such as increasing the thickness of the TMJ cartilage, decreasing the volume of subchondral bone, flattening the

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condyle, osteophyte formation, even a serial of degenerative changes. Estrogen replacement can restore most of the changed histomorphometric parameters (Yasuoka *et al.*, 2000). It was found that estradiol affected chondrocyte cell growth, DNA, and proteoglycan synthesis in a biphasic manner depending on its concentration (Cheng *et al.*, 2003). Estrogen caused temporomandibular disorders (TMD) with induction of the proinflammatory cytokines e.g. IL-1 β , IL-6 and IL-8 (Yun *et al.*, 2008). All these results indicate that estrogens have the potential to modify the metabolic activity of TMJ, and this has received additional support from the existence of estrogen receptors (ERs) in TMJ. Both ER α and ER β have been detected in both the male and female rat TMJ (Yu *et al.*, 2009).

In addition, the bone mass in elderly men was more significantly related to estradiol levels than to testosterone (Slemenda *et al.*, 1997; Szulc *et al.*, 2001). Therefore, estrogen replacement therapy (ERT) is proposed to prevent bone loss in males as well as in females (Rochira *et al.*, 2000; Ockrim *et al.*, 2003). Estrogen, nevertheless, was one of the hormonal risk factors of benign prostatic hyperplasia and prostate cancer in men (Harkonen and Makela, 2004; Soronen *et al.*, 2004).

Phytoestrogens are a group of non-steroidal polyphenolic plant metabolites that induce biological responses and can mimic or modulate the action of endogenous estrogens, often by binding to estrogen receptors (Shutt and Cox, 1972). The committee on the toxicity of chemicals in food, consumer product and the environment (2003 report) claimed that the principal phytoestrogen-classes are isoflavones, lignans and coumestans.

According to folk medicine, coconut juice (*Cocos nucifera* L., Arecaceae), contains several active compounds with various therapeutic properties. Phytoestrogens seem to have beneficial effects that include enhancement of cutaneous wound healing. Among the constituents of phytoestrogens are flavonoids like kaemferol, isoflavones, luteoline and apigenin (Spilkova and Hubik, 1992; Havsteen, 2002). Our previous study demonstrated that YCJ had a wound healing effect (Sayoh *et al.*, 2008) and indicated that one of the active compounds is likely to be a member of the flavonoid group. The aim of this study, therefore, was to investigate any role for YCJ, that presumably contains phytoestrogen, for its possible beneficial effects on delaying osteoporosis in male rats.

2. Materials and Methods

2.1 Plant material

Throughout this study, young coconut juice was collected from 6 month old fruit from one area (100 square yards) in the Tungngai district, Hat Yai, Songkhla, Thailand. A large quantity of YCJ was lyophilized and the powder was kept at -30°C until used. This powder was freshly reconstituted and prepared for oral feeding every day. The complete

description of YCJ, including its preparation and administration, is provided in a previous publication (Radenahmad *et al.*, 2006).

2.2 Animals

All animals used were adult two-month old male Wistar rats weighing approximately 230g. The animals were housed in a controlled environment at 25±1°C with an illumination schedule of 12h light/12h dark. Rats had unrestricted access to standard pellet food and water. The study was approved by the Ethics Committee on Animal Care and was carried out in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Prince of Songkla University.

2.3 Experimental design

There were five groups of rats (6 per group) included in this study. The first group consisted of orx rats, the second group consisted of sham-operated rats, the third group consisted of orx rats injected intraperitoneally with exogenous estrogen (2.5 μ g/kgBW of estradiol benzoate, EB) 5 days a week for two weeks. The fourth group consisted of orx rats that received YCJ (100 ml/kgBW/day) and the fifth group was sham-operated rats receiving YCJ (100 ml/kgBW/day). The dose of YCJ in this study was based on the one reported in our earlier study and in which dose standardization and optimal administration was set (Radenahmad *et al.*, 2006). The dose of EB in this study was also the same as our earlier study (Radenahmad *et al.*, 2006). In this study, the administration of EB and YCJ was started two weeks after ovariectomy was performed. Rats belonging to the first and second groups were forced fed with deionized water.

2.4 H and E staining of the mandibular condyle cartilage and morphological analysis

After sacrifice, the mandibular condyle were dissected, fixed in 10% formalin and decalcified with 10% formic acid solution for 2 weeks. Radiography was used to determine the completeness of decalcification. After routine histological laboratory procedures, tissues were blocked in paraffin and mid-sagittal sections of 5 μ m were cut and stained with H and E for histological examination. Three sections from the middle part of each joint were selected for this study. The total cartilage thickness and four separated layer of mandibular condyle were investigated using light microscopy at x200 magnification. Briefly, a standardized vertical framework of 3 lines, 175 μ m apart, was created and positioned over the midpoint region of each image (Talwar *et al.*, 2006). The total cartilage thickness and each layer of mandibular condylar cartilage were measured as described by Carlson (1994). Linear measurements were made of the constituent zone heights, as well as the total cartilage thickness (Figure 5), and measurements were performed using an Image Pro Plus

program (DP11, Olympus SZX 12, Japan). Slides were viewed blindly. Readings from the three sections from each rat were then added and the average was determined.

2.5 Serum estradiol and testosterone

All the rats were sacrificed on the first day of the fifth week. Their serum was collected for estradiol (E2) and testosterone measurements using the chemiluminescent immuno assay (CIA) technique, as set by the manufacturer (ECLIA, Modular E 170C, Estradiol II 03000079 122, Roche, Germany) and (ECLIA, Modular E 170C, testosterone 11776061 122, Roche, Germany), respectively. Details of the CIA technique have been explained in our previous publication (Radenahmad *et al.*, 2009).

2.6 Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis and the Mann-Whitney U-tests available in the statistical program SPSS version 11.5. The Altman's nomogram for sample size calculations was used to determine the sample size. Random selection of the microscopic fields was achieved using a computer generated list of random numbers (Excel version 5.0). Results were reported as means \pm SEM. $P<0.05$ was considered to be significantly different.

3. Results

3.1 Serum E2 levels

Our results showed that the sham or orx rat groups that received YCJ had serum E2 levels that were not significantly ($P>0.05$) different from the orx or sham operated groups (Figure 1). A significant difference ($p<0.05$) was observed when the serum E2 level of the orx+EB group was compared with the other groups.

3.2 Serum testosterone levels

Our results showed that the sham group or the sham group receiving YCJ had serum testosterone levels significantly higher than the other groups at $p<0.01$ and $p<0.05$ respectively. Feeding YCJ to the sham group caused a significant reduction of the testosterone level (Figure 2).

3.3 Effects of YCJ on histological changes of mandibular condylar cartilage

There was no evidence that orx by itself had any effect on the measured thicknesses of mandibular condylar cartilage of the male rats. After 14 days of treatment with YCJ, the total mandibular condylar cartilage thickness particularly the hypertrophic zone was thickest in the sham+YCJ group and thinnest in the sham group (Figures 3-5). However only the sham+YCJ group showed any significant change, an

increase ($p<0.01$), in total cartilage thickness compared to the others. The thickness of the hypertrophic zone of the sham+YCJ group was significantly higher than that of sham and orx groups at $p<0.01$ and the orx+YCJ was also significantly higher than the orx or the orx+EB groups (Figures 3, 4, 5).

4. Discussion

There is increasing evidence to support the idea of using phytoestrogens as an alternative strategy to HRT since they can also produce additional health benefits. These include a reduction in the risk of HRT-induced cancers and the prevention of osteoporosis and cardiovascular diseases. However, none of these suggestions has been thoroughly tested, particularly the use of YCJ to prevent osteoporosis.

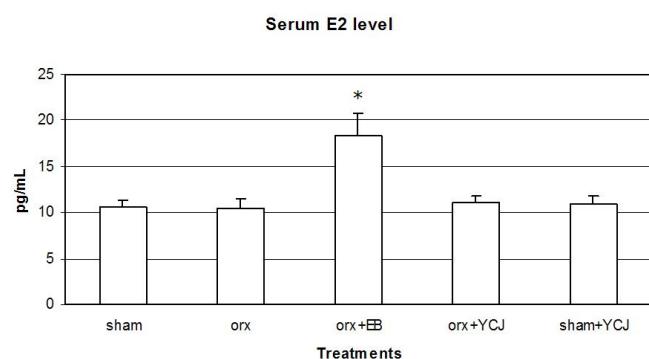


Figure 1. Serum estradiol (E2) level (mean \pm SEM) in the five groups examined. orx: orchidectomized group; orx+EB: orchidectomized group receiving estradiol benzoate; orx+YCJ: orchidectomized group receiving young coconut juice; sham+YCJ: sham-operated group receiving young coconut juice. $n=6$ in each group. * $p<0.05$ compared with sham, orx, orx+EB, orx+YCJ and sham+YCJ groups.

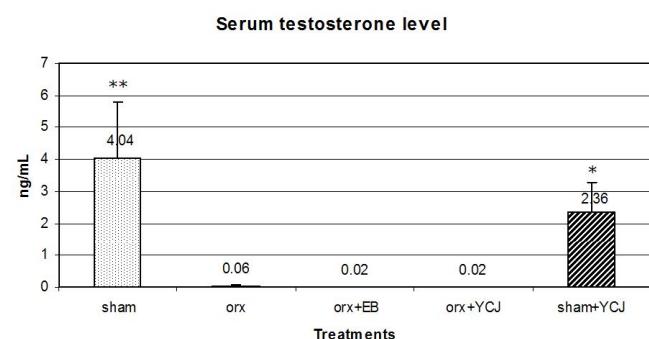


Figure 2. Serum testosterone level (mean \pm SEM) in the five groups examined. orx: orchidectomized group; orx+EB: orchidectomized group receiving estradiol benzoate; orx+YCJ: orchidectomized group receiving young coconut juice; sham+YCJ: sham-operated group receiving young coconut juice. $n=6$ in each group. * $p<0.05$, ** $p<0.01$ compared with orx, orx+EB and orx+YCJ groups.

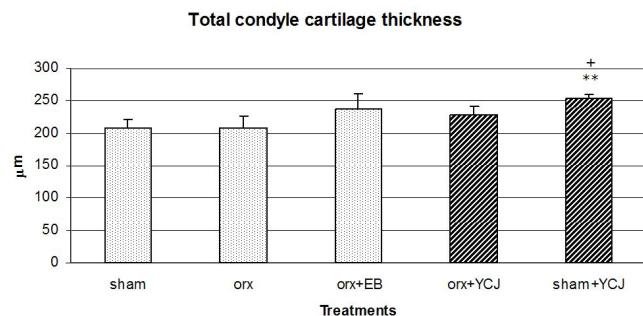


Figure 3. Condyle cartilage thickness of mandible of sham+YCJ group was significantly higher than sham and orx groups at $p<0.01$ and $p<0.05$ respectively. orx+EB: orchidectomized group receiving estradiol benzoate; orx+YCJ: orchidectomized group receiving young coconut juice; sham+YCJ: sham-operated group receiving young coconut juice. $n=6$ in each group. ** $p<0.01$ compared with sham group, + $p<0.05$ compared with orx group.

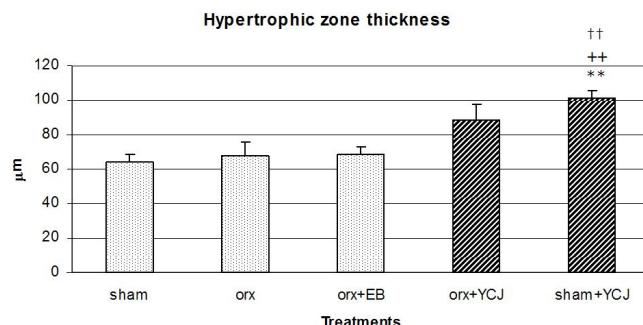


Figure 4. Hypertrophic zone thickness of condyle cartilage of the mandible of sham+YCJ group was significantly higher than sham and orx groups at $p<0.01$. orx+EB: orchidectomized group receiving estradiol benzoate; orx+YCJ: orchidectomized group receiving young coconut juice; sham+YCJ: sham-operated group receiving young coconut juice. $n=6$ in each group. **, +, †† $p<0.01$ compared with sham, orx and orx+EB groups respectively.

It has been established that the level of osteoporosis can be assayed by examining the hypertrophic zone of mandibular condylar cartilage (Cassorla *et al.*, 1984; Nasatzky *et al.*, 1994; Gray, 1989). The thinner the hypertrophic zone the greater the osteoporosis. The purpose of this study was to determine whether YCJ, a product that has been shown to contain estrogen-like compounds (Punghmatharith, 1988), had any beneficial effects on preventing osteoporosis in sham/orx rats.

In the present study, the levels of E2 and testosterone in orx rats were not increased after forced feeding with YCJ. In orx rats, rats' testis were removed, so there was no source of testosterone at all. After administration of coconut juice, each component was individually metabolized in the body of rats. In addition, "phytoestrogen" has a weak affinity in the CIA assay compared to exogenous estrogen (in this case,

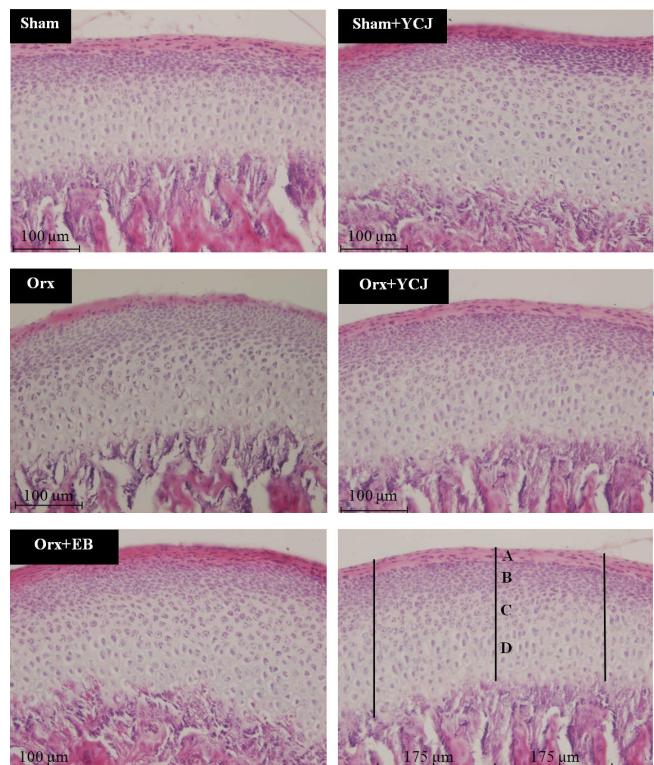


Figure 5. Mid-sagittal sections of condylar cartilage of rat mandible (magnification $\times 400$). The sections were stained with hematoxylin-eosin. Total cartilage thickness and the hypertrophic zone were thickest in the sham+YCJ group. The bottom right picture show the four zones of condylar cartilage: (A) the articular zone, (B) the proliferation zone, (C) the maturation zone, (D) the hypertrophic zone. A standardized overlay of 3 vertical lines, approximately 175 μm apart was used for all histologic specimen measurements. Sham: sham-operated group; Sham+YCJ: sham-operated group receiving young coconut juice; orx: orchidectomized group; orx+YCJ: orchidectomized group receiving young coconut juice; orx+EB: orchidectomized group receiving estradiol benzoate.

EB). Therefore, it showed as a very low concentration when measured by CIA.

The level of testosterone level was reduced in sham rats receiving YCJ. In sham-operated rats, the testis were intact and when the rats were force fed with YCJ ("sham+YCJ" group), testosterone level was decreased (2.36 ng/mL) approximately 40% compared with sham group (4.04 ng/mL). This could be explained by production of a sex steroid cascade. Testosterone is changed to 17β -estradiol (E2) by an aromatase enzyme, therefore when the rats received "extra" estrogen (from YCJ), this might have had a feedback effect to the adrenal gland to reduce testosterone production.

The cartilage layer of mandibular condylar cartilage is composed of four zones: the articular, proliferative, maturation and hypertrophic zones (Luder *et al.*, 1988; Mizoguchi *et al.*, 1996). The proliferative zone has mesenchymal cells

distributed as chondrocyte precursors for the underlying zones (Bibb *et al.*, 1992). Differentiated chondrocytes are found in the maturation and hypertrophic zones and are the main sources of the subchondral bone (Furseth, 1996). In this study, we examined the benefits of the oral intake of YCJ on the growth of mandibular condylar cartilage by measuring the width of each zone and of the total mandibular condylar cartilage.

Previous studies in female rats has revealed that estrogen reduced mandibular condylar cartilage (May *et al.*, 1999). Furthermore, the thickness of the proliferative and hypertrophic zones were increased in ovx rat (Yamashiro and Yakan-Yamamoto, 1998; Turner *et al.*, 1994) and the thickness of the hypertrophic zone was reduced following administration of E2 (May *et al.*, 1999). Estrogen has been shown to protect against osteoporosis in postmenopausal women, but many reports have indicated that in both male and female, estradiol serum levels were higher in TMD patients than in normal healthy controls (Landi *et al.*, 2004, 2005). Thirty percents of women over the age of 40 who suffered from TMD had a medical history of receiving exogenous estrogen (LeResche *et al.*, 1997). Altogether, this data indicated that either endogenous or exogenous estrogens reduced mandibular condyle cartilage thickness.

In contrast, the present study revealed that sham-operated male rats, after 14 days of treatment with YCJ, had a higher total cartilage thickness and hypertrophic zone thickness compared to other groups. E2 level was highest in orx+EB group though. This might be due to different estrogen receptors in the mandibular condylar cartilage. TMJ has both ER α and ER β receptors (Yu *et al.*, 2009) and endogenous or exogenous estrogens are specific to ER α rather than to ER β . In contrast, the phytoestrogens of YCJ react specifically with ER β rather than ER α receptors (Kuiper *et al.*, 1998; Sayoh *et al.*, 2008). Interestingly, the increased thickness was better displayed in the group receiving YCJ, as compared to the group receiving EB. This indicates that the receptors for the estrogen-like components of YCJ have a greater affinity, as compared to those for EB. Kuiper and colleagues have found that phytoestrogens exhibit a great affinity for ER β (Kuiper *et al.*, 1998). Subsequent studies by our group on the immunohistochemistry of both ER receptors are needed to confirm this.

Using radioimmunoassay techniques, Punghmatharith found that 1 ml of coconut juice contained 2.45 pg of 17 β -estradiol and also other sex hormone-like substances (see details in Punghmatharith, 1988). Results from thin-layer chromatography studies also confirmed that YCJ contained substances similar to estrone, such as 17 β -estradiol, and β -sitosterol (Punghmatharith, 1988). Such studies also found that subcutaneous injection of an ethereal extract of YCJ reconstituted at a dose equivalent to 7,500 mL of young coconut juice/kgBW/day for 3 consecutive days significantly increased the uterine wet weight of immature rats. Furthermore, YCJ has been found to contain β -sitosterol as well as other sterols like stigmastatrienol, stigmasterol,

fucosterol etc. β -sitosterol is structurally related to animal cholesterol, and could possibly act as a precursor of sex steroids (Moghadasian, 2000). In the present study, β -sitosterol and stigmasterol (plant sterols known as precursors of steroid hormones *in vivo*) could be responsible for the estrogenic effect of the YCJ. This remains to be confirmed by our group in subsequent studies. The effects of YCJ on cancellous bone mass in male and female rats are being conducted.

5. Conclusion

The novel data provided in this report demonstrate that biomarkers for osteoporosis in living animals are influenced by compounds derived from a plant (namely YCJ) with possible estrogen-like ingredients. This could hopefully be shown in the future to have positive implications for the prevention and treatment of osteoporosis in elderly men.

Conflict of interest

The authors hereby declare that there is no conflict of interest in this manuscript.

Acknowledgements

Appreciation to Dr Brian Hodgson for assistance with English. This study was supported by the Department of Anatomy, Faculty of Science; Department of Conservative Dentistry, Faculty of Dentistry; and Nutraceutical and Functional Food Research and Development Center, Prince of Songkla University, Hat Yai, Thailand.

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