



Original Article

Effects of young coconut juice on the numbers of argyrophil endocrine cells in the gastrointestinal tract of male rats: Novel preliminary findings

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Abstract

Apart from calcium itself, there are many factors including vitamin D and estrogen, that play important roles in bone formation. Hormones, especially estrogen, used for replacement therapy is highly effective at reducing the rate of bone loss and can also replace lost bone in postmenopausal women. Estrogen replacement therapy has been proposed to prevent bone loss in males as well as in females. Estrogen, however, has been considered to be one of the hormonal risk factors of benign prostatic hyperplasia and prostate cancer and also other side effects. With this background, in the present study, young coconut juice (YCJ), that is known to contain the phytoestrogen, β -sitosterol, was investigated for its possible beneficial effects on delaying osteoporosis using a male orchidectomized rat model, and as a replacement for estrogen replacement therapy. In the present study we used the Grimelius stain which is a broad endocrine cell marker, especially in the gastrointestinal (GI) tract to quantify the argyrophil endocrine cells and if possible to relate this to reflex GI functions, e.g. calcium absorption, GI motility etc. that might have an influence on osteoporosis. There were five groups of rats (6 per group) included in this study. The first group consisted of sham-operated rats, the second group consisted of orchidectomized (orx) rats, and the third group consisted of orx rats injected intraperitoneally with exogenous estrogen (2.5 μ g/kgBW of estradiol benzoate, EB) five 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) for two weeks. After sacrifice, the GI tract including stomach, small and large intestines were removed, fixed and paraffin embedded for routine H&E and Grimelius silver staining. Most of the argyrophil cells were dispersed in the mucosa, particularly in the basal mucosa and were generally round or spindle shaped and occasionally spherical. There was a regional distribution of the argyrophil endocrine cells in all regions of the GI tract: stomach, duodenum, jejunum, ileum and colon, and that of the orx group had the lowest numbers. Estradiol benzoate (EB) injection and YCJ feeding in the orx group reversed this phenomenon in all regions of GI tract except for the colon. The sham+YCJ group had the highest number of argyrophil cells. Regression correlation analysis between the argyrophil cell numbers and serum estrogen and testosterone levels were R^2 0.0054 and 0.0355, respectively. The changes in the density of the GI argyrophil cells in the orchidectomized rats in the present study may contribute to the GI function abnormality e.g. GI motility, GI calcium absorption, GI hormone production and secretion etc. frequently encountered in patients with andropausal or elderly osteoporosis. Such impairment of calcium and probably other minerals in the orx rats could be reversed by exogenous estrogen e.g. estradiol benzoate (EB) supplement or by phytoestrogen e.g. YCJ feeding as preliminarily detected by the numbers of argyrophil endocrine cells in this study.

Keywords: young coconut juice, *Cocos nucifera*, osteoporosis, grimelius staining, gastrointestinal tract

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1. Introduction

Osteoporosis has become a major worldwide health-care problem because of the rapidly aging population. In Thailand, the prevalence of osteoporosis among Thai men was 4.6 and 12.6 per cent for the lumbar spine and femoral neck, respectively (Pongchaiyakul *et al.*, 2006). Androgens play a very important role in building the skeleton of young adults and help to prevent bone loss and osteoporosis in aging men. Furthermore, in elderly men, bone mass has been related to estrogen levels rather than to testosterone.

Bone is a “dynamic tissue” that is remodeled throughout life (Longo *et al.*, 2011). Before middle age, bone resorption by osteoclasts is well balanced with bone formation by osteoblasts. After menopause, estrogen deficiency causes bone loss due to bone resorption exceeding bone formation resulting in osteoporosis (Nussey and Whitehead, 2001). Apart from calcium, there are many other factors including vitamin D and estrogen that play an important role in bone formation. Estrogen replacement therapy has, therefore, been proposed to prevent bone loss in males as well as in females. Estrogen, however, has been considered to be one of the hormonal risk factors of benign prostatic hyperplasia and prostate cancer and also other side effects. With this background, in the present study, young coconut juice (Y CJ), that is known to contain the phytoestrogen, β -sitosterol (Rattanaburee *et al.*, 2014), was investigated for its possible beneficial effects on delaying osteoporosis using an orchidectomized male rat model, and as a replacement for estrogen replacement therapy.

Silver stains have played an important role in identifying various cell types including endocrine cells particularly those in the gastrointestinal (GI) tract. GI endocrine cells are dispersed in the epithelia and glands of the digestive tract. They synthesize a variety of GI hormones that have an influence on the functions of the alimentary tract. Therefore, changes in their density might have impacts on GI functions e.g calcium absorption, one of the important factors related to the development of osteoporosis. We have previously reported a decrease of GI argyrophil endocrine cells in a post-menopausal osteoporotic rat model. There was less reduction in rats fed Y CJ for a maximum of 2 weeks (Sayoh *et al.*, 2008). Using the same model, two preliminary studies were also conducted on the effects of Y CJ on increasing the condylar cartilage thickness and mandibular cancellous bone in orchidectomized rats by our group (Yusuh *et al.*, 2010; Suwanpal *et al.*, 2011). The purpose of this study was, therefore, to observe any changes of the regional distribution and number of GI argyrophil endocrine cells in orchidectomized (orx) male rats receiving Y CJ supplement.

2. Materials and Methods

2.1 Plant material

Throughout this study, young coconut juice was

collected from 6-month old fruits from one area (100 square yards) in the Tungngai District, Hat Yai, Songkhla, Thailand. A large quantity of Y CJ was freeze dried 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 Y CJ, including its preparation and administration, has been 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 230 g. The animals were housed in a controlled environment at $25\pm 1^{\circ}\text{C}$ with an illumination schedule of 12 h light/12 h dark. Rats had unrestricted access to standard pellet food and water. The study was approved by the Ethics Committee on Animal Care, Reference 07/51, and was carried out in accordance with the *Guiding Principles for the Care and Use of Research Animals* promulgated by the 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 sham-operated rats, the second group consisted of orx rats, and the third group consisted of orx rats injected intraperitoneally with exogenous estrogen ($2.5\text{ }\mu\text{g/kgBW}$ of estradiol benzoate, EB) five days a week for two weeks. The fourth group consisted of orx rats that received Y CJ (100 mL/kgBW/day) and the fifth group was sham-operated rats receiving Y CJ (100 mL/kgBW/day) for two weeks. The dose of EB and Y CJ in this study was also the same as in our earlier study (Radenahmad *et al.*, 2006). In this study, the administration of EB and Y CJ was started two weeks after orchidectomy was performed. Rats belonging to the first and second groups were force fed with deionized water.

2.4 Grimelius staining of the gastrointestinal (GI) tract and morphological analysis

The Grimelius stain was modified from Grimelius and Wilander (1980). Briefly, sections had the paraffin removed, then were hydrated with acidulated water for 5 minutes. Sections were then treated with a 0.1% silver solution and incubated in an oven at 60°C for 1 hour. Sections were then placed in a hot reducing solution at 60°C for 5 minutes and rinsed in 3 changes of hot acidulated water for 2 minutes each. Sections were then transferred to a 0.1% silver solution at 60°C for 10 minutes followed by again rinsing in hot acidulated water at 60°C for 5 minutes then in another hot solution for another 10 minutes. Finally, sections were rinsed in tap water, dehydrated in 95% and 100% alcohol, cleared in xylene and covered with Permount. Argyrophil cells were stained black and the cytoplasm was light yellow to gold.

Slides were viewed by a researcher who had no

knowledge of their origins. Readings from ten randomized areas per section and 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 out 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 statis-

tical program SPSS version 16. 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 significant.

3. Results

3.1 Effects of YCJ on histological changes of argyrophil endocrine cells in the GI tract of male rats

The distribution and frequency of argyrophil endocrine cells in the regions of the gastrointestinal tract (GI) were studied using the Grimelius silver stain. Argyrophil cells were detected in the GI tract with various frequencies. Most of the argyrophil cells were dispersed in the mucosa, particularly in the basal mucosa and, were generally round or spindle shaped and occasionally spherical (Figures 1 and 2). There was a regional distribution of the argyrophil endocrine cells in all regions of the GI tract: stomach, duodenum,

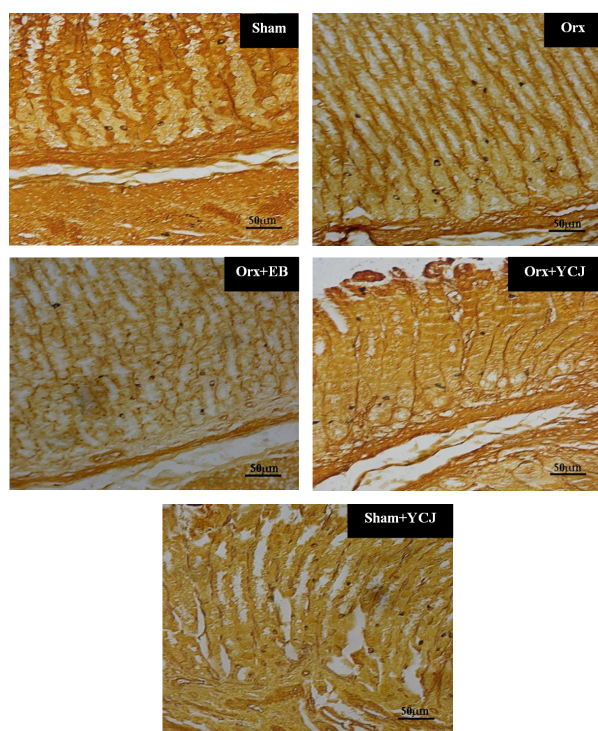


Figure 1. Argyrophil endocrine cells in the pylorus of sham, orx, orx+EB, orx+YCJ and sham+YCJ. Argyrophil granules appeared as black large spots due to silver staining. Most of argyrophil cells were dispersed in the mucosa, particularly in the basal mucosa of the pylorus. In the pylorus, argyrophil cells were highest in the sham+YCJ group and that of orx group was lowest. Grimelius silver staining. Sham: sham-operated group; 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.

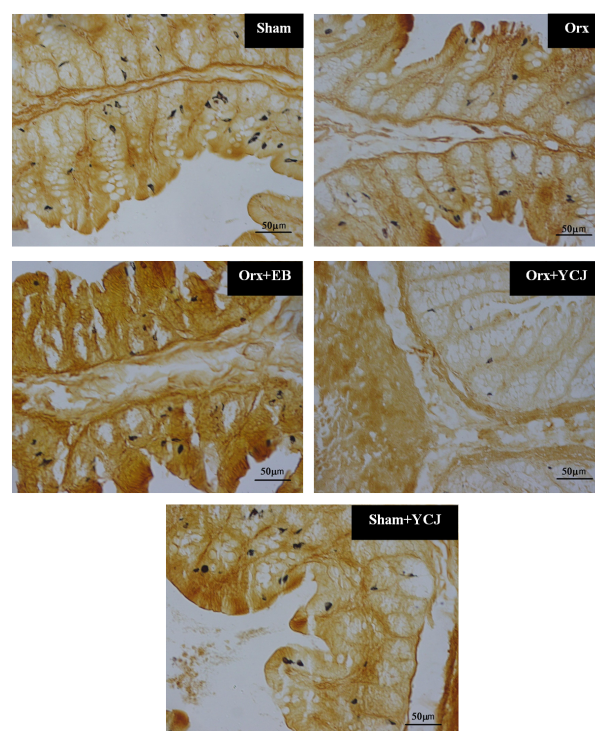


Figure 2. Argyrophil endocrine cells in the colon of the sham, orx, orx+EB, orx+YCJ and sham+YCJ. Argyrophil granules appeared as black large spots due to silver staining. Most of argyrophil cells were dispersed in the mucosa, particularly in the basal mucosa of the colon. Numbers of argyrophil cells were highest in the sham+YCJ group and, surprisingly, that of orx+YCJ group was lowest. Grimelius silver staining. Sham: sham-operated group; 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.

jejunum, ileum and colon, and that of the orx group had the lowest numbers (Figure 3 and Table 1). Estradiol benzoate (EB) injection and YCJ feeding in the orx rats reversed this phenomenon in all regions of GI tract except the colon. It was a surprise, to find that the sham+YCJ group had the highest number of argyrophil cells. Regression correlation analysis between the argyrophil cell numbers and serum estrogen and testosterone levels were R^2 0.0054 and 0.0355, respectively (Figures 4 and 5).

4. Discussion

It is generally accepted that osteoporosis is a metabolic and hormonal disorder that is clearly related to lowered

estrogen levels (O'Toole *et al.*, 1985; Riggs 2002). Also osteoporotic patients and/or animals show quite different feeding habits when compared with their normal counterparts (Thomas, 2003). Silver staining techniques have been regarded as a general method for detecting GI endocrine cells and Grimelius positive cells are classified as argyrophil cells (Grimelius, 1968; Grimelius and Wilander, 1980). The GI endocrine cells can in general be divided into two types, one is round to spherical shaped closed type cells that are located in the stomach regions, and the other is spherical to spindle shaped open type cells that are situated in the intestinal regions. Our results are in agreement with the studies of Ku *et al.* (2004) and Wang *et al.* (2010). Argyrophil cells are a type of endocrine cell, regarded as the anatomical units

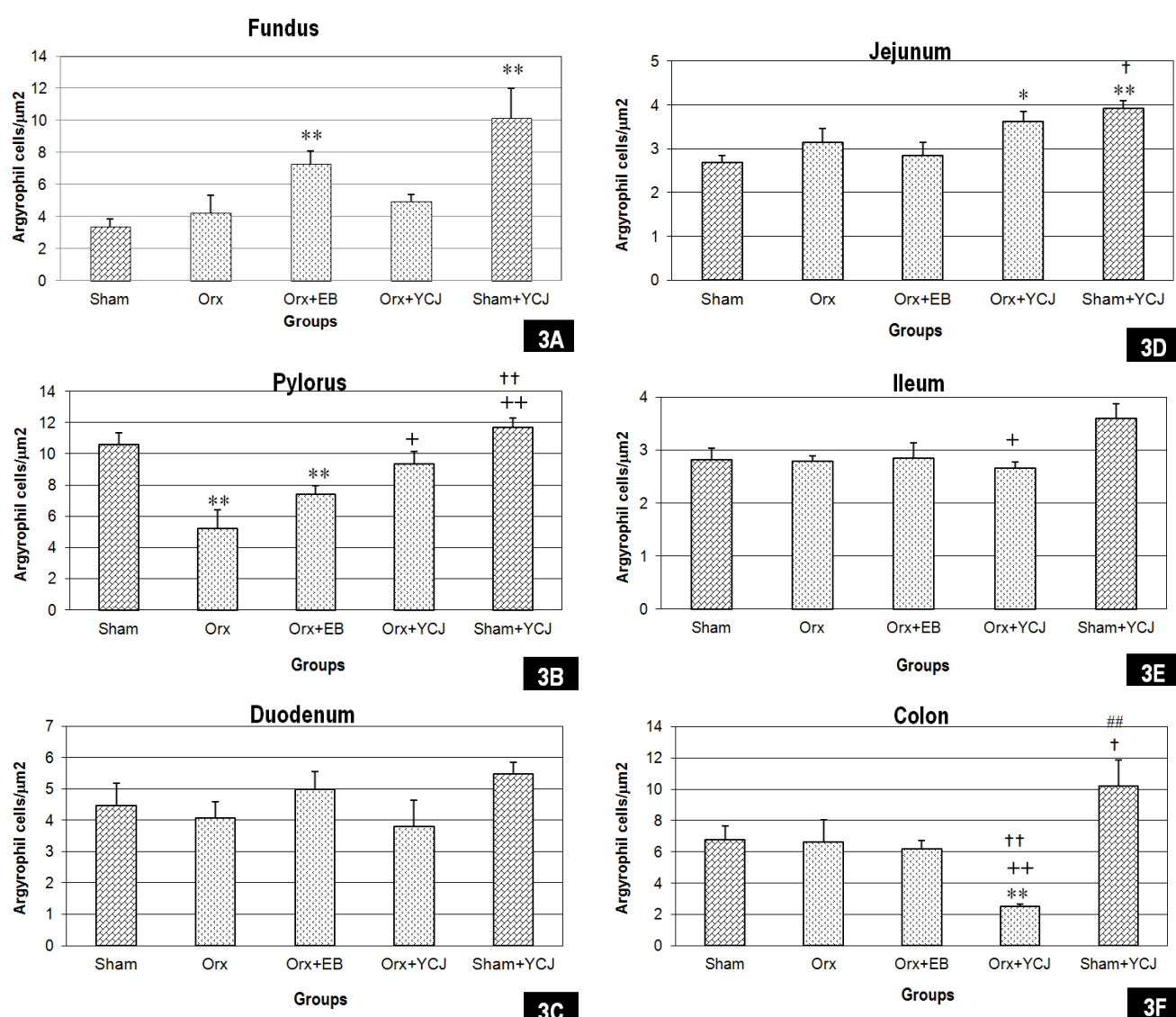


Figure 3. Number of argyrophil cells in the GI tract and their changes after orchidectomy and YCJ feeding. Sham: sham-operated group; orx: orchidectomized group; orx+EB: orchidectomized group receiving estradiol benzoate; orx+YCJ: orchidectomized group receiving young coconut juice; sham+YCJ: sham-operated group receiving young coconut juice. $n = 6$ in each group. * $p < 0.05$, ** $p < 0.01$ compared with sham group; † $p < 0.05$, †† $p < 0.01$ compared with orx group; ‡ $p < 0.05$, ‡‡ $p < 0.01$ compared with orx+EB group; ## $p < 0.01$ compared with orx+YCJ group.

Table 1. Number of argyrophil cells (cell/ μm^2) in various parts of the gastrointestinal (GI) tract of YCJ treated rats compared with control groups.

| | Sham | Orx | Orx+EB | Orx+YCJ | Sham+YCJ |
|----------|------------------|-------------------------------|-------------------------------|-------------------------------------|-----------------------------------|
| Fundus | 3.37 \pm 0.47 | 4.24 \pm 1.10 | 7.3 \pm 0.77 ^{aa} | 4.93 \pm 0.45 | 10.13 \pm 1.89 ^{aa} |
| Pylorus | 10.60 \pm 0.76 | 5.20 \pm 1.19 ^{aa} | 7.43 \pm 0.53 ^{aa} | 9.35 \pm 0.81 ^b | 11.70 \pm 0.60 ^{bb,cc} |
| Duodenum | 4.47 \pm 0.72 | 4.07 \pm 0.51 | 4.98 \pm 0.56 | 3.80 \pm 0.83 | 5.48 \pm 0.35 |
| Jejunum | 2.68 \pm 0.16 | 3.14 \pm 0.32 | 2.85 \pm 0.29 | 3.62 \pm 0.24 ^a | 3.93 \pm 0.17 ^{a,c} |
| Ileum | 2.82 \pm 0.22 | 2.78 \pm 0.11 | 2.85 \pm 0.29 | 2.65 \pm 0.13 ^b | 3.60 \pm 0.27 |
| Colon | 6.77 \pm 0.89 | 6.63 \pm 1.43 | 6.18 \pm 0.54 | 2.52 \pm 0.14 ^{aa,bb,cc} | 10.22 \pm 1.66 ^{c,d} |

Note: Sham: sham-operated group; 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. ^{a,aa} p<0.05, p<0.01 compared with sham group respectively; ^{b,bb} p<0.05, p<0.01 compared with orx group respectively; ^{c,cc} p<0.05, p<0.01 compared with orx+EB group respectively; ^d p<0.05, compared with orx+YCJ group.

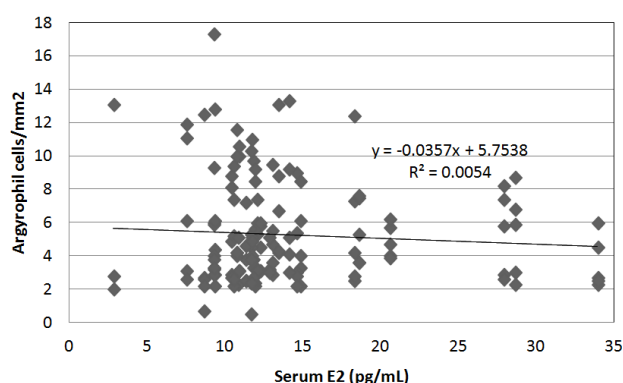


Figure 4. Negative correlation between number of argyrophil cells in GI tract and serum E2 level in the five groups examined.

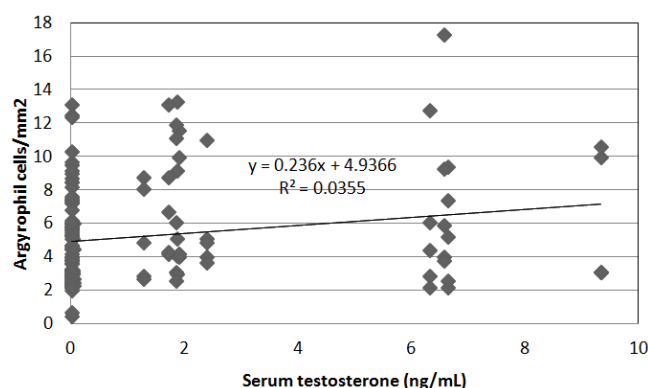


Figure 5. Positive correlation between the number of argyrophil cells in the GI tract and the serum testosterone level in the five groups examined.

responsible for production of gut hormones (Ku *et al.*, 2004) that control GI movement, GI gland secretions, and epithelial cell growth in the mucosal layer (Wang *et al.*, 2010). Therefore, changes in their density would reflect changes in tissue's capacity to produce these hormones (El-Salhy and Sitohy, 2001).

Argyrophil cells have been found in the GI tract e.g. stomach, duodenum, jejunum, ileum and colon in many species of animals (Cetin, 1992; Bodegas *et al.*, 1997; Fonseca *et al.*, 2002; Rindi *et al.*, 2004; Kostiukevich, 2004). Our study, is in agreement with these results; argyrophil cells were found in the gastrointestinal mucosa of Wistar male rats from the stomach to the colon (Figures 3A-3F). The present study shows that the largest number of argyrophil cells was in the stomach, particularly the pylorus, of the sham-operated group and the numbers were less in the colon, duodenum, jejunum and ileum, respectively. Therefore, from the stomach to the large intestine, the density of the argyrophil cells distribution is "U-shaped". This pattern resembles that of the distribution of endocrine cells in the small intestine of the

rats studied by Josephson and Altmann (1973) and also in the African ostrich (Wang *et al.*, 2010).

In the present study, the general distribution of the argyrophil cells in the GI tract of the orx group showed quite similar patterns compared to that of the sham operated. As a result of orchidectomy, argyrophil cells significantly decreased throughout the entire GI tract particularly in the pylorus of the stomach. In the duodenum, ileum and colon, argyrophil cells in the orx group were decreased compared to those of the sham group but the significant differences were not recorded. It was unexpected to find that the argyrophil cells in the jejunum and fundus of the orx group had a similar distribution and number compared to those of the sham group. EB injection restored the number in the orx rats except for in the fundus and pylorus. The argyrophil cell number in the pylorus of the orx+EB group was significantly lower ($p<0.01$) and in the fundus was significantly higher ($p<0.01$) when compared with that of the sham group. In the pylorus, in contrast to the EB supplement, YCJ feeding restored that phenomenon in orx rats and the number was

significantly increased ($p < 0.05$) when compared with that of the orx group. In contrast, in the colon, YCJ feeding in the orx group dramatically reduced that number. However, YCJ feeding of the sham group significantly increased the number of argyrophil cells in the fundus, pylorus, jejunum and colon compared with other groups. In the duodenum and ileum argyrophil cells in the sham+YCJ group dramatically increased compared to those of the sham group but significant differences were not observed. It is not clear how the exogenous estrogen, EB or phytoestrogen-sterols in the YCJ react with estrogen receptors of each part of the GI tract. Subsequent studies on the immunohistochemistry of estrogen receptors have been designed to confirm this.

The changes of the argyrophil numbers above were confirmed by regression correlations with both hormones: serum estrogen and testosterone levels. The regression correlations indicated that the number of argyrophil cells had a negative correlation ($R^2 = 0.0054$) with the estrogen level, and that of the argyrophil cells had a positive correlation ($R^2 = 0.0355$) with the testosterone level. These phenomena might be due to the characteristics of the steroid hormones compared to the plasma-binding proteins. Estradiol binds to the sex hormone-binding globulin (SHBG) with a much lower affinity than testosterone. Adult males have about one-half as much circulating SHBG as females, so free testosterone is about 20 times greater in males than in females. In addition, the total (bound and unbound) concentration of testosterone is about 40 times greater in males. Testosterone itself lowers SHBG levels and increases the amount of free testosterone in the blood, whereas 17β -estradiol raises the SHBG levels in the blood (Devlin, 2011).

The present study confirms our previous studies on cartilage and bone of the temporomandibular joint (TMJ) of the same male rat model. After 14 days of treatment with YCJ, the total mandibular condylar cartilage thickness particularly in the hypertrophic zone was thickest in the sham+YCJ group and thinnest in the sham group. That thickness of the orx+EB and orx+YCJ groups was higher than that of the sham or orx groups, even though the significances were not observed (Yusuh *et al.*, 2010). After 14 days of treatment with YCJ, the mandibular cancellous bone measured as a percentage of the bone volume was significantly thicker in the sham+YCJ, orx+YCJ and orx+EB groups than that of the sham and orx groups (Suwanpal *et al.*, 2011). Therefore, the changes of argyrophil cells in the present study might support the changes of mandibular condylar cartilage and mandibular cancellous bone by, for example, increasing calcium absorption in the GI tract, through an increase of the GI releasing hormones.

YCJ has been found to contain β -sitosterol as well as other sterols (Rattanaburee *et al.*, 2014; Rujilarai and Sitaruno, 2009). β -sitosterol is structurally related to animal cholesterol, and could possibly act as a precursor of the sex steroids (Moghadasian, 2000). In the present study, β -sitosterol and stigmasterol (plant sterols known to be precursors of steroid hormones *in vivo*) could be responsible

for the estrogenic effect of the YCJ. This remains to be confirmed.

However, YCJ may still work in male animals in which the testes are still intact and secrete testosterone. It can be seen that the testosterone levels in the sham and sham+YCJ groups are much higher than in either the orx, orx+EB, or orx+YCJ groups (Yusuh *et al.*, 2010). The effect of YCJ may have synergistic effects with the presence of testosterone or some unknown substance secreted by the testis. It is known that in elderly men the testosterone levels decline gradually but slowly during their last stage of life. It means that the elderly men still have some certain level of testosterone in their blood and the treatment with YCJ (sham+YCJ group) may have an agonistic effect with endogenous testosterone as seen in Figure 5 and Table 1. This would explain why the number of argyrophil cells in the sham+YCJ group is higher than that of either the sham or the other control groups. This result is in agreement with our previous work on the mandibular condylar cartilage and the cancellous bone of TMJ using the same rat model (Yusuh *et al.*, 2010; Suwanpal *et al.*, 2011) as mentioned above.

Estrogenic compounds express their activities by binding to different estrogen receptors (ERs), $ER\alpha$ and $ER\beta$. $ER\alpha$ dominates in a few specific tissues whereas $ER\beta$ is expressed in other tissues including bone and the GI tract (Gustafsson, 1999). While endogenous or exogenous estrogens are specific to $ER\alpha$ rather than to $ER\beta$, YCJ containing the phytoestrogen, β -sitosterol, reacts specifically with $ER\beta$ rather than $ER\alpha$ (Kuiper *et al.*, 1998; Sayoh *et al.*, 2008). Furthermore, results from previous studies in knock out mice indicate that those two forms of ER may have two different biological roles (Devlin, 2011). For example, $ER\alpha$ and $ER\beta$ have coexpression activity in neurons e.g. $ER\alpha$ is implicated in synaptic plasticity, while $ER\beta$ is involved with neural cell differentiation (Devlin, 2011). Thus, $ER\alpha$ and $ER\beta$ might have different biological effects in each region of the GI tract. Subsequent studies on the immunohistochemistry of both estrogen receptors are being conducted to confirm this. Specific patterns of change in other bone types: axial bones and long bones are being prepared for further investigations. Altogether, these results indicate that feeding YCJ could replace current HRT for use to limit osteoporosis in elderly men or men with hypogonadism.

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