Young coconut juice increased number of calbindin and vitamin D receptor cells via estrogen receptors in gastrointestinal tract of orchidectomized rats

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SUMMARY

Adult male rats were orchidectomized, and their gastrointestinal tract was stained immunohistochemically with specific antibodies against calbindin (CB), a calcium-binding protein and vitamin D receptor (VDR), two essential factors in calcium absorption and bone formation. Compared to normal rats, the number of CB-immunoreactive and VDR-immunoreactive cells in the gastrointestinal tract were significantly reduced in orchidectomized rats, but was restored to normal by injecting these rats with estradiol benzoate or by feeding with young coconut juice (YCJ), which was found to be not dose-related. In an attempt to find out if the osteoporosis-protective effects of YCJ were due to the binding of the YCJ active component(s) with estrogen receptors, ERα and ERβ, anti-ERα and ERβ were detected immunohistochemically, and a significant correlation was detected between CB-/VDR-reactive cells vs. ERα-/ERβ-reactive cells. Immunohistochemical profiles showed significant correlations between CB-/VDR-immunoreactive cells vs. ERα-/ERβ-immunoreactive cells. The results suggest that YCJ may be as effective as estradiol benzoate in reducing osteoporosis, probably as a selective estrogen receptor moderator.

Key words: *Cocos nucifera* L – *Arecaceae* – Calbindin – Vitamin D receptor – Osteoporosis – Orchidectomy

ABBREVIATIONS

CB	Calbindin
EB	Estradiol benzoate
ERα	Estrogen receptor-α
ERβ	Estrogen receptor-β
GI tract	Gastrointestinal tract
-ir	-immunoreactive
ORx	Orchidectomized
SERM	Selective estrogen receptor modulator
VDR	Vitamin D receptor
YCJ	Young coconut juice

INTRODUCTION

Osteoporosis is considered a harmful condition. Estrogen replacement therapy has been proposed to prevent bone loss in both females and males (Tu et al., 2018; Rochira et al., 2008). However, this therapy has been implicated with an increased

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risk of breast cancer in women and prostate cancer in men (Bitzer et al., 2008; Chen et al.,2015).

The gastrointestinal (GI) tract is responsible for many functions, e.g., nutrient, electrolyte and fluid, including calcium absorption. Calcium homeostasis is regulated by parathyroid hormone and vitamin D, and calcium plays a vital role in many crucial mechanisms, including cell differentiation, intracellular signaling, and bone formation (Kirchhoff and Geibel, 2006). Our previous study used Grimelius stain, a broad endocrine cell marker especially in the GI tract, to quantify the argyrophilic endocrine cells and, when applicable, to reflex GI functions, e.g., calcium absorption, GI motility, which might have an influence on osteoporosis in male rats. We have previously reported a decrease of GI argyrophilic endocrine cells in both male and female osteoporotic rat models, and young coconut juice (YCJ) feeding reversed this phenomenon (Sayoh et al., 2008; Radenahmad et al., 2014). Two preliminary studies were also conducted using the same model on the effects of YCJ on increasing the condylar cartilage thickness and mandibular cancellous bone in orchidectomized (ORx) rats by our group (Yusuh et al., 2010; Suwanpal et al., 2011). Later, we found that YCJ has estrogenic effects to prevent bone loss in ovariectomized rats (Yooprasert et al., 2015).

Calbindin-D9k (CaBP-9k), hereafter will be referred to as calbindin (CB), is a cytosolic vitamin D-dependent calcium-binding protein that strongly binds calcium, and is expressed in various tissues, such as the pituitary gland, uterus, placenta, intestines, kidneys, and bones (Choi and Jeung, 2008). It regulates cytoplasmic calcium concentration by facilitating calcium uptake to maintain intracellular Ca^{2+} concentrations below 10^{-7} mol/L. This regulatory role is essential to prevent cell death from free calcium toxicity (Hong and Jeung, 2013) and premature apoptotic cell death (Barboza et al., 2015).

Vitamin D plays an essential function in calcium homeostasis, and, in its active form of 1,25-dihydroxy, vitamin D3 is a potent stimulator of active intestinal calcium absorption through the active transcellular pathway, one of the essential pathways that carry out intestinal calcium uptake and the passive paracellular pathways through tight junctions, through the enterocytes, then to the body (Christakos et al., 2011).

Since calcium is an essential mineral in bones, intestinal calcium absorption through vitamin D and calbindin (Lee et al., 2003) plays a significant role in calcium uptake through the alimentary canal. Altogether, with this background, the aim of the present study was, therefore, to find out if YCJ could prevent osteoporosis in orchidectomized male rats by detecting calbindin and vitamin D receptor (VDR) cells in the GI tract, in addition to their effect on the ovariectomized rats (Jiangsakul et al.,2015). The number of ERα- and ERβ-immunoreactive cells were also investigated to determine if YCJ could act through these receptors.

Thus far, the active ingredient(s) of YCJ has not been identified, although it was suspected to be β-sitosterol (Rattanaburee et al., 2014) and probably to be isoflavone group due to its wound healing property. It was found that YCJ at 100 mL/kg BW/day caused unfavorable glycogen deposition in the liver in ovariectomized rats (Radenahmad et al., 2012 unpublished data). Therefore, in the present study, three lower doses (10, 20, and 40 mL/kg BW/day) were designed to investigate whether it effectively prevents osteoporosis via preventing relevant cells in the GI tract of orchidectomized rats.

MATERIALS AND METHODS

YCJ preparation

A large volume of YCJ was collected from Khlong Hoi Khong district, Hat Yai, Songkhla, Thailand. It was then dried, and the powder formed was kept at -30°C until used. The powder was freshly reconstituted and prepared daily for oral intake. A complete description of YCJ, including its preparation and administration, is provided in our previous publication (Radenahmad et al., 2006).

Animals

Adult male Wistar rats (8-month-old and 250-300 g) were purchased from Mahidol University, Salaya campus. The animals were maintained on standard food pellet housed in a room free from any source of chemical contamination, artificially illuminated (12h dark/light cycle), and under

controlled thermal (25 ± 1°C) and humidity (50 ± 5%) conditions, at the Animal House Laboratory, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of Prince of Songkla University and the National Institutes of Health (NIH publication 86-23 revised 1985. The protocol was approved under the license number 01/59).

Experimental design

After an acclimatization period of 1 week, the animals were divided into seven groups (10 rats/ group) and treated for ten weeks as follows: group 1, normal baseline control (NC) animals, were sacrificed on the first day of the experiment without any treatment; group 2, sham-operated rats (SC), received reverse-osmosis water; groups 3, orchidectomized rats, received reverse-osmosis water (OC); group 4, ORx rats, intraperitoneally injected three days a week with 2.5 µg/ kg BW estradiol benzoate, EB (OE). The dose of EB given was the same as in our previous studies (Radenahmad et al., 2009; 2012); groups 5, 6, and 7, ORx rats orally treated with YCJ at 10, 20, and 40 mL/kg BW/day (OJ10, OJ20, and OJ40), respectively (Table 1a). At the end of the treatment period, all animals were sacrificed, and the stomach (body and fundus), small intestines (duodenum, jejunum, ileum), and colon of each

Table 1. Animal grouping, antibodies, concentration, and manufacturer used for GI sections of each rats.

a. Animal grouping (10 rats per group).

Group	Treatment
NC	Normal (normal control)
SC	Sham-operated, received reverse-osmosis water (sham control)
OC	Orchidectomized, received reverse-osmosis water (ochidectomized control)
OE	Orchidectomized, and injected with estradiol benzoate (EB, 2.5 µg/kg BW) 3 days a week, for 10 wks
OJ10	Orchidectomized, received YCJ at 10 mL/kg BW/d for 10 wks
OJ20	Orchidectomized, received YCJ at 20mL/kg BW/d for 10 wks
OJ40	Orchidectomized, received YCJ at 40mL/kg BW/d for 10 wks

b. Description of slides for immunostaining for each rat GI regions.

Section No.	Staining
1-2	H & E staining, for histological orientation
3-4	Anti-calbindin antibody (N0142, Sigma-Aldrich, USA)
5-6	Anti-VDR antibody (P3088, Sigma-Aldrich, USA)
7-10	For sections 7 and 8, mouse anti-estrogen receptor α (aa-120-170) antibody (MAB447, Chemicon international, USA) and for sections 9 and 10, estrogen receptor β antibody (PA1-310B, Thermo Fisher Scientific, USA)
11-12	Immunostaining, omitting primary antibodies (negative control), one for each antibody

c. Dilutions of each antibody for each organ of GI tract (body and fundus of stomach; duodenum, jejunum, and ileum of small intestine; colon).

	Antibodies			
Organs	**VDR**	**Calbindin (CB)**	**ER-alpha**	**ER-beta**
Body	1:500	1:500	1:100	1:50
Fundus	1:500	1:500	1:100	1:50
Duodenum	1:500	1:100	1:500	1:100
Jejunum	1:500	1:100	1:500	1:75
Ileum	1:500	1:50	1:800	1:75
Colon	1:500	1:100	1:500	1:200

animal were removed, fixed with 10% neutral formalin, paraffin processing, sectioning, and immunohistochemical staining.

Immunohistochemistry

Twelve 5 µm-thick sections from each block were prepared for Hematoxylin and Eosin (H&E) stain and immunostaining (Table 1b and 1c). For immunostaining, the glass slides were coated with the poly-L-lysine solution. Sections of uterus and ovary from normal female rats were used as positive controls for ERα, and ERβ immunostaining, respectively, and the staining process was performed according to the method previously described (Radenahmad et al., 2009; 2012). Details of all antibodies, concentrations, and manufacturers used for GI sections of each rat can be consulted in Tables 1b and 1c.

Quantitative analysis of immunoreactive cells

The total number of immunoreactive cells from the stomach, small intestines, and colon were counted under light microscopy with 40x magnification power. Two blinded observers performed the counting on ten random fields of each slide using an image analysis system (Samba microscopic image processor; Samba Technologies, Meylan, France). Readings from 3 sections pertaining to each antibody were averaged and expressed as the mean number of immunoreactive cells/mm^2.

Statistical analysis

Shapiro-Wilk test was applied to test the normal distribution. Statistical analysis was performed using the One-way ANOVA, followed by the LSD test available in the statistical program SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). Altman's nomogram was used for calculations of sample size. Random selection of the microscopic fields was achieved using a computer-generated list of random numbers (Excel version 5.0). Results were expressed as mean ± SEM. A $p<0.05$ value was considered significant.

RESULTS

Figs. 1 and 2 depict examples of immunoreactive cells for CB, VDR, ERα, and ERβ in the GI specimens of the control groups. The most colorful staining for each antibody, the highest and the lowest

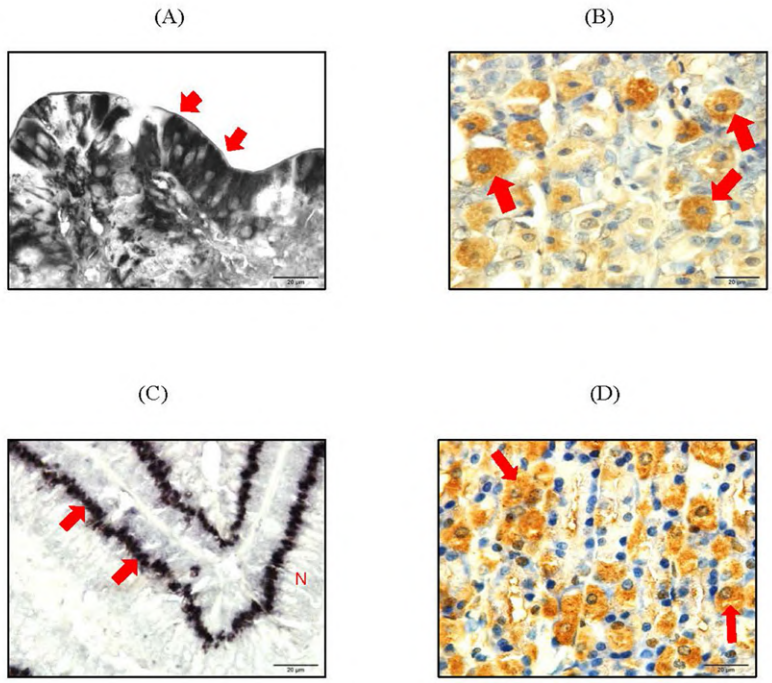


Fig. 1.- Examples of cells that were CB- (A), VDR-(B), ERα-(C) and ERβ-(D) immunoreactive cells in different parts of the gastrointestinal tract of the control groups (N = nucleus). **A)** CB-immunoreactive cells in the cytoplasm of the colon epithelium. **B)** VDR-immunoreactive cells in the cytoplasm of parietal cells of the stomach. **C)** ERα-immunoreactive cells in the villi of enterocyte of duodenum. **D)** ERβ-immunoreactive cells in the cytoplasm of parietal cells of the stomach. Scale bars: 20 µm.

number of immunoreactive cells of each organ, is shown in Table 2a. The CB and VDR were localized in parietal cells of the fundus and the body of the stomach. A positive reaction was observed in the cytoplasm, but not in the nucleus of stomach parietal cells, in enterocytes of the small intestine villi, and colonocytes of the colon. The ERα and ERβ were observed in the cytoplasm, not in the nucleus of the stomach's parietal cells. Surprisingly, a positive reaction was located in the perinuclear cytoplasm of enterocytes of villi throughout the small intestines and colon. In

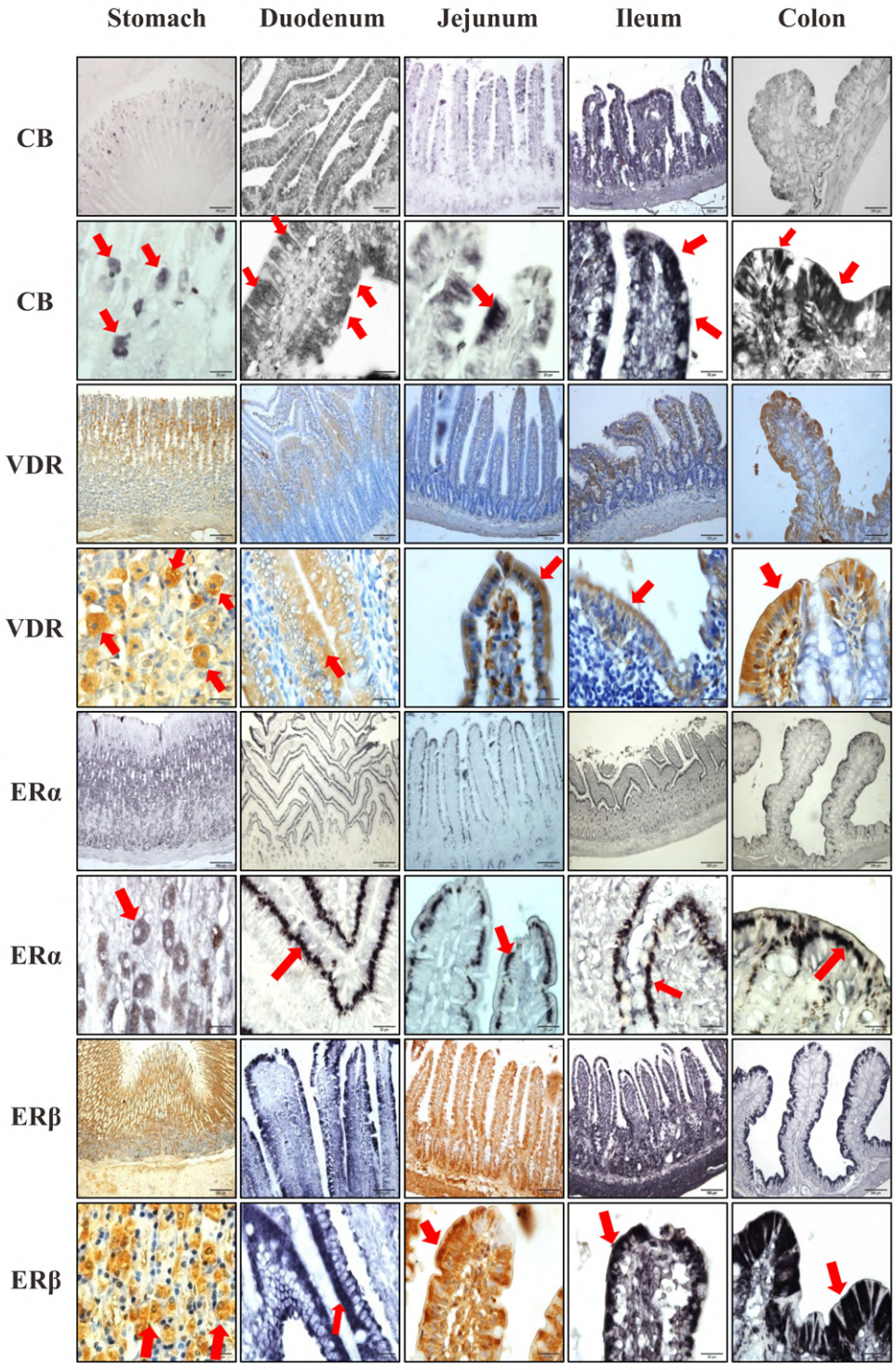


Fig. 2.- Photographs showing immuno-histochemistry of CB-, VDR-, ERα- and ERβ- immunoreactive cells from the stomach, duodenum, jejunum, ileum and colon of the control groups. CB-, VDR-, ERα- and ERβ-immunoreactivity was observed in the cytoplasm of parietal cells of stomach, cytoplasm of enterocytes of small intestines and colon. The same scale is used for all pictures of each row. The first row of each antibody is at 20x magnification, while the next row of the same antibody is at 100x magnification. Red arrows indicate immunoreactive cells for each antibody. Scale bars: 100 µm, 20 µm respectively.

addition, ERα immunoreactivity was found at the nucleus of the intestinal gland of the jejunum and the ileum. The ERα immunoreactivity was highest in the cytoplasm of the colonocytes. In contrast, The ERβ immunoreactivity was highest in the cytoplasm of parietal cells of the stomach.

In addition, ERα-ir cells were found at the nucleus of intestinal gland of the jejunum and the ileum. The intensity of ERα immunoreactivity was the strongest in the cytoplasm of the colonocytes. In contrast, The ERβ-ir cells were detected strongest in the cytoplasm of the parietal cells of the stomach.

Fig. 3A depicts the number of immunoreactive CB (CB-ir) cells in various regions of the GI tract. In the NC and SC groups, the immunoreactive cells were observed at high frequency in all regions. Following orchidectomy, the numbers of CB-ir cells significantly dropped in almost all regions except that in the duodenum. The values increased to normal when the ORx rats were treated with EB, except in the ileum. With YCJ treatments, the number of immunoreactive cells increased but was not dose-related. The number of CB-ir cells of all three OJ groups were not significantly different when compared to each other, nor when compared with the control groups, except in the ileum, where all three OJ groups were significantly lower than that of the NC and SC groups. The number of CB-ir cells in the OJ10 group (fundus) was, nevertheless, significantly higher than that of the NC, SC, OC, and OE groups.

In summary, the total number of CB-ir cells per mm^2 of seven groups examined was found highest in the duodenum (189.95±41.59), and the lowest in the fundus of the stomach (3.07±1.20) (Table 2b).

Table 2a. Description of each antibody for the most beautiful staining, the highest and the least number of positive staining of 6 regions of GI tract examined.

Antibodies	The most colorful staining	The highest number of positive staining	The least number of positive staining
CB	Colon	Duodenum	Stomach (Fundus)
VDR	Stomach	Colon	Stomach (Fundus)
ERα	Duodenum	Ileum	Stomach (Fundus)
ERβ	Colon	Stomach (Fundus)	Duodenum

Table 2b. Total number of immunoreactive cells of each antibody in 6 regions of gastrointestinal (GI) tract.

Antibodies	Organs Regions	Total number of Immunoreactive Cells (per mm^2) in 7 groups examined
CB	Fundus of Stomach Body of Stomach Duodenum Jejunum Ileum Colon	3.07 ± 1.20 3.96 ± 1.02 189.95 ± 41.59 56.47 ± 18.38 65.65 ± 17.47 91.14 ± 16.87
VDR	Fundus of Stomach Body of Stomach Duodenum Jejunum Ileum Colon	66.90 ± 12.71 81.77 ± 10.98 292.83 ± 72.31 249.84 ± 54.21 150.60 ± 34.97 340.97 ± 32.02
ERα	Fundus of Stomach Body of Stomach Duodenum Jejunum Ileum Colon	166.73 ± 23.08 235.77 ± 37.41 724.15 ± 126.27 580.43 ±93.02 760.92 ± 133.64 417.38 ±69.77
ERβ	Fundus of Stomach Body of Stomach Duodenum Jejunum Ileum Colon	542.58 ±46.54 718.89 ± 70.65 239.26 ± 34.84 378.68 ± 58.44 495.46 ± 73.47 242.19 ± 35.20

In contrast, the number of VDR-ir cells, compared with CB-ir cells region by region, increased in all six regions of the GI tract. In the NC and SC groups, VDR-ir cells were observed at a high number in all regions. Following orchidectomy, the numbers of VDR-ir significantly dropped in all six regions examined, then was reversed when treated with EB or YCJ treatments. With YCJ treatments, the increase of the immunoreactive cells was not dose-related (Fig. 3B).

Among the three doses of YCJ treatments, the numbers of VDR-ir in the OJ20 group were highest in the fundus and body of stomach, duodenum and jejunum, while VDR-ir cell numbers in the OJ10 group were highest in the colon. The number of VDR-ir cells in the OJ20 and OJ40 groups were comparable to those of the control (NC and SC groups). Surprisingly, there was no significant difference in the duodenum when the OJ groups were compared with the control

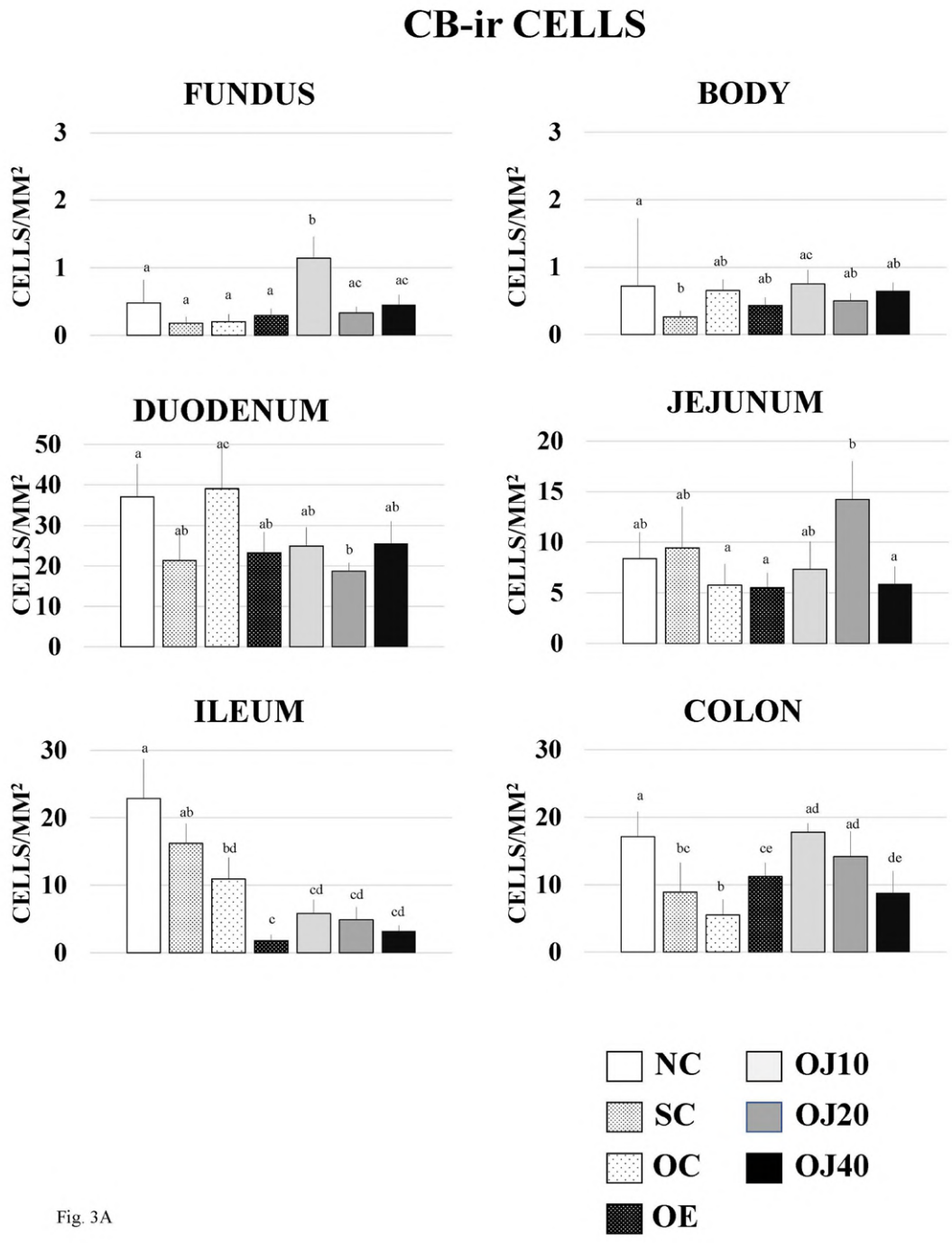


Fig. 3A

Fig. 3.- 3A) Number of CB-immunoreactive cells from the fundus, body, duodenum, jejunum, ileum, and colon. Columns superscript with different letters are significantly different at $p < 0.05$.

groups, neither when each group was compared (Figure 3B). In summary, Table 2b shows the total number of immunoreactive cells per mm^2 in the six regions. The highest was found in the colon (340.97±32.02), and the lowest in the fundus of the stomach (66.90±12.71).

Fig. 3C shows that, unlike VDR-ir cells, ERα-ir cells were found in more significant numbers in all six regions of the GI tract examined in this study. Nevertheless, the number of immunoreactive cells in the control and the YCJ treatment groups were like that of VDR-ir cells. In the NC and SC groups, like VDR-ir, the positive cells were observed at high frequency in all regions. Following orchidectomy, the numbers of ER-ir cells significantly dropped in all six regions. The number increased when the ORx rats were treated with EB or YCJ treatments. With YCJ treatments, the numbers of the immunoreactive cells did not increase in a

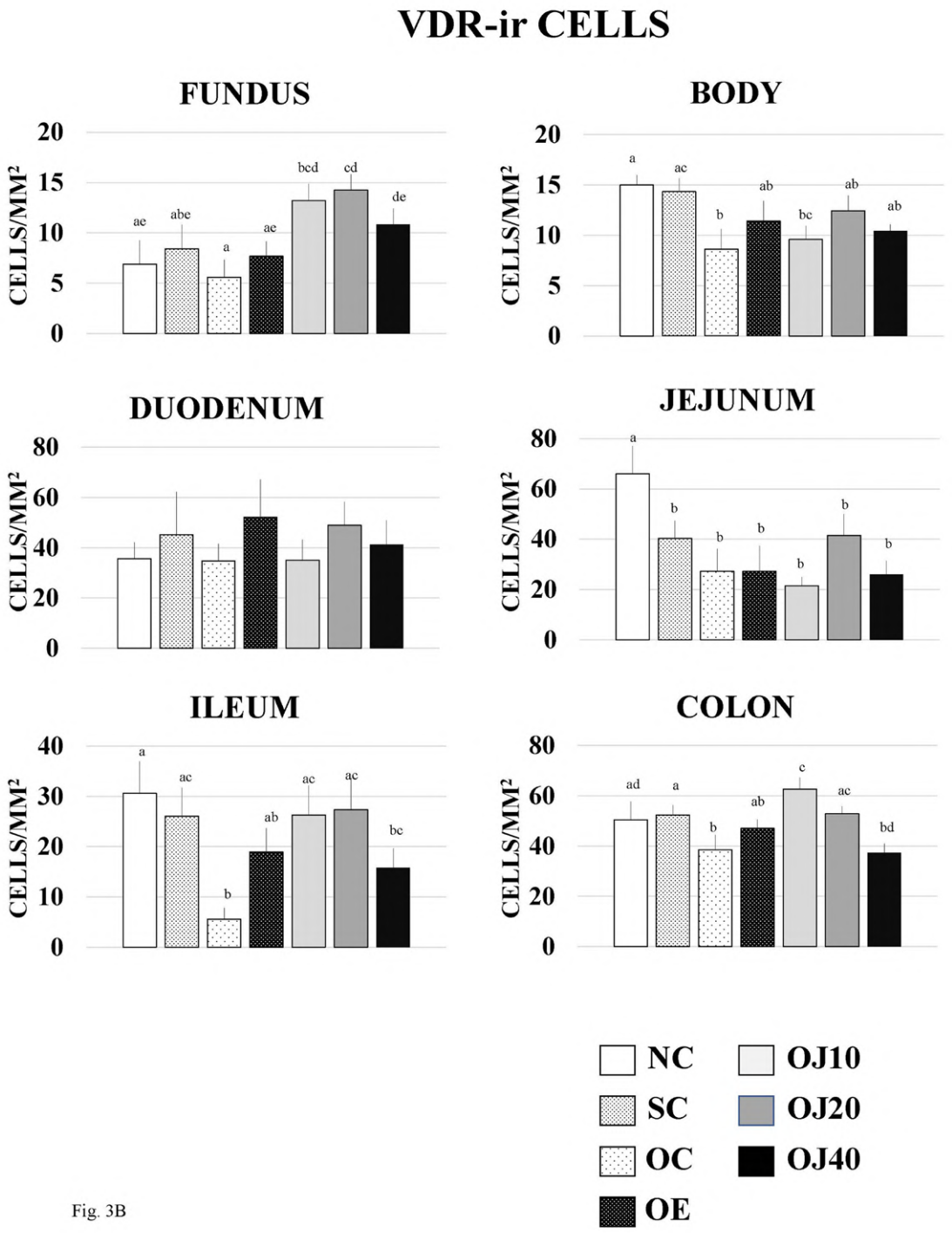


Fig. 3B

Fig. 3.- 3B) Number of VDR-immunoreactive cells from the fundus, body, duodenum, jejunum, ileum, and colon. Columns superscript with different letters are significantly different at $p < 0.05$.

dose-related manner. The OJ10 group showed the highest ERα-ir cell number in almost all regions understudies, while the OC group had the lowest number in all six regions examined. Surprisingly, there was no significant difference between the number of ERα-ir cells in the body of the stomach and the jejunum of OJ groups and control groups, neither when the groups were compared to each other. In summary, the total number of ERα-ir per mm^2 in the six regions examined was highest in the ileum (760.92±133.64), and the lowest in the fundus of the stomach (166.73±23.08) (Table 2b).

Table 2b shows that the number of ERβ-ir was higher than ERα-ir in all six regions of the GI tract examined in this study but was comparable to a number of ERα-ir cells in the control and the YCJ treatment groups. Fig. 3D shows that, following orchidectomy, the number of ERβ-ir significantly dropped in all six regions examined except in the

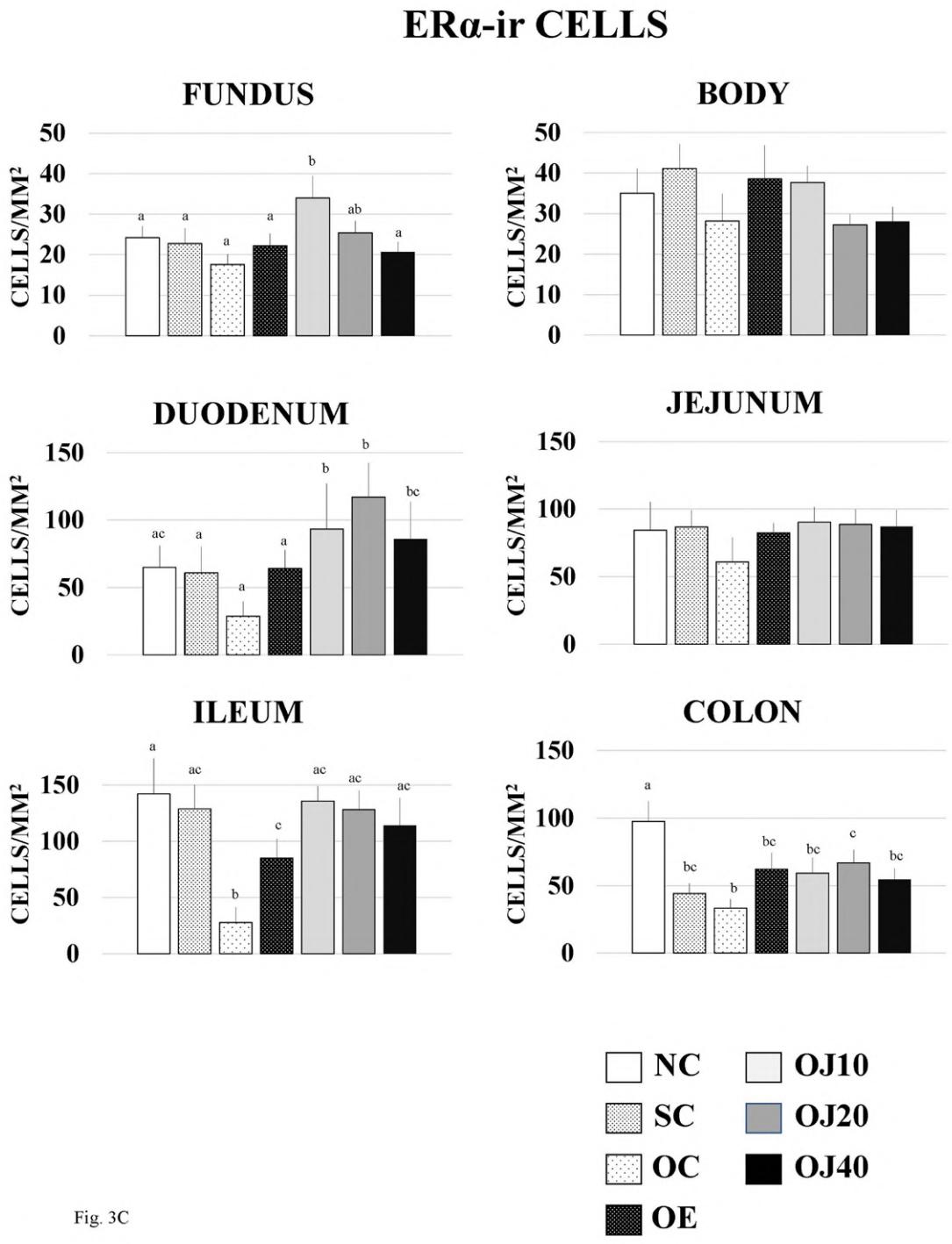


Fig. 3C

Fig. 3.- 3C) Numbers of ERα-immunoreactive cells from the fundus, body, duodenum, jejunum, ileum, and colon. Columns superscript with different letters are significantly different at $p < 0.05$.

body of the stomach, then increased when the ORx rats were treated with EB or YCJ treatments. With YCJ treatments, the numbers of the immunoreactive cells increased comparably with the control groups (NC and SC). In addition, in the body of the stomach, there was an insignificant difference between the OJ groups and the control groups, neither when each group was compared to the other. On the contrary to the other three antibodies, CB, VDR, and ERα, the total number of ERβ-ir per mm^2 in the six regions examined were highest in the body of the stomach (718.89±70.65) and the lowest in the duodenum (239.26±34.84) (Table 2b).

To find out if the numbers of CB-ir, VDR-ir were correlated to each other and with the numbers of ERα- and ERβ-ir cells, the number of CB-ir cells were plotted against those of VDR-, ERα- and ERβ-ir cells in the same animals, regardless of groups

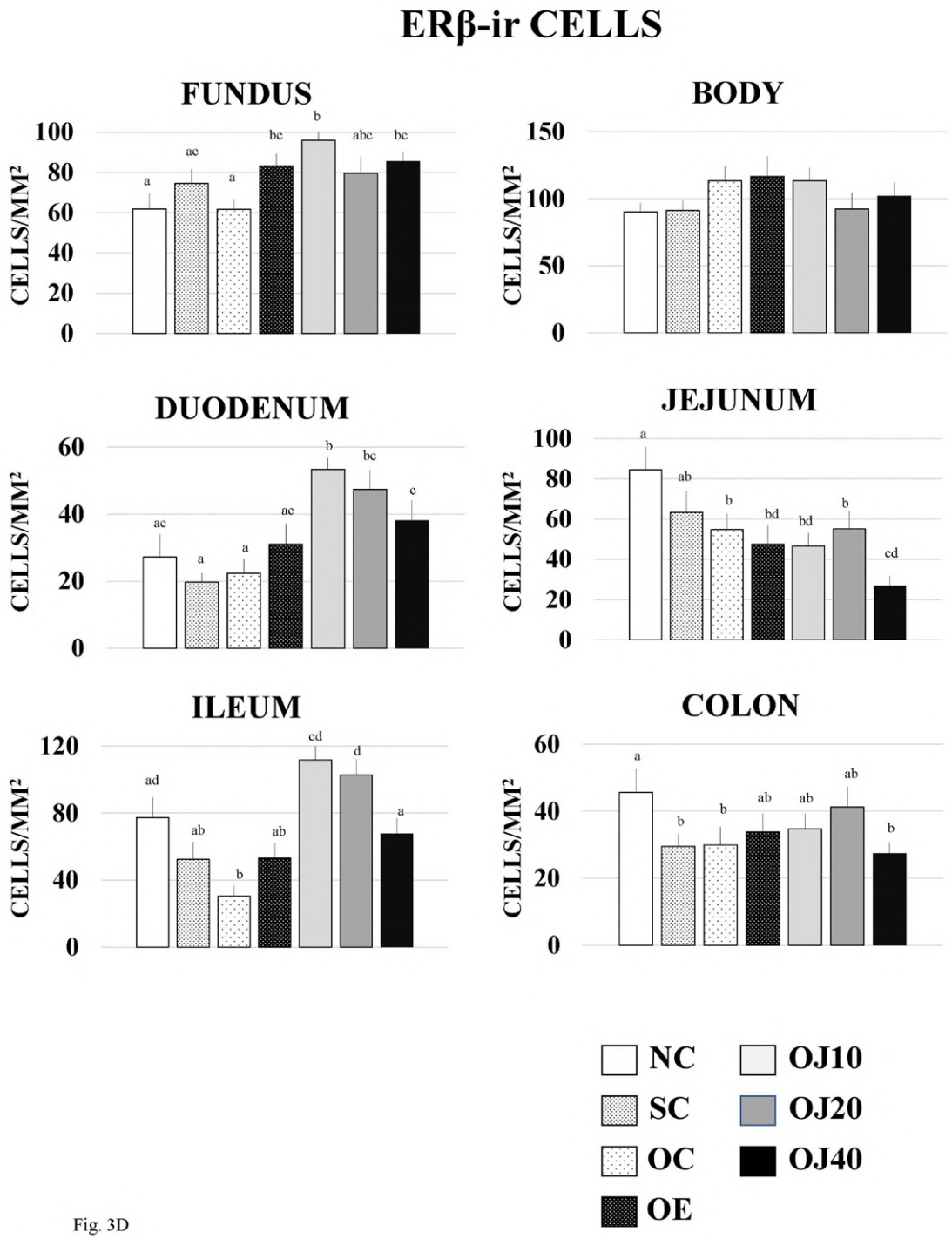


Fig. 3D

Fig. 3.- 3D) Number of ERβ-immunoreactive cells from the fundus, body, duodenum, jejunum, ileum, and colon. Columns superscript with different letters are significantly different at $p < 0.05$.

(Fig. 4). Likewise, the number of VDR-ir cells was plotted against those of ERα- and ERβ-ir cells.

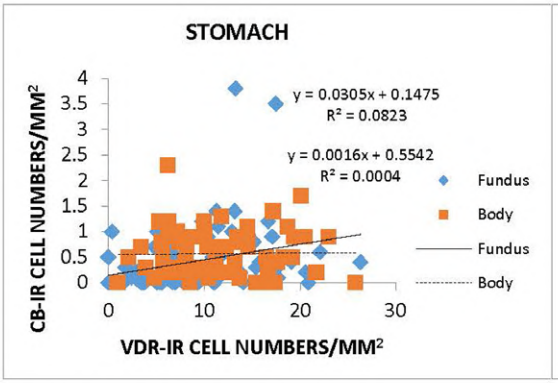
The results revealed that the number of CB-ir cells was positively correlated with the number of VDR-ir cells in all regions of the GI tract (stomach, small intestine, and colon). The number of CB-ir cells were correlated positively with the numbers of ERα- and ERβ-ir cells, except in the ileum. The numbers of CB-ir correlated negatively with ERα-ir cells. The number of VDR-ir positively correlated with that of ERα- and ERβ-ir in all regions of the GI tract.

DISCUSSION

For centuries, YCJ has been used for various therapeutic purposes, but not for the prevention of

Fig. 4

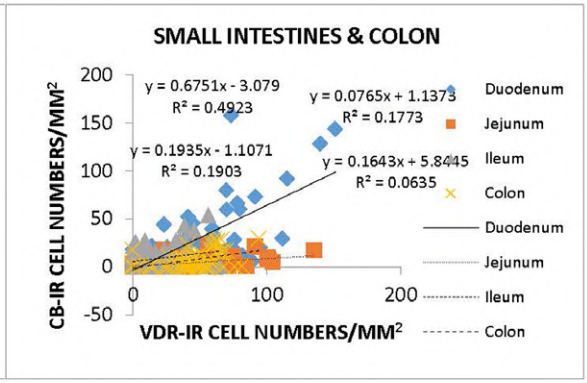
(a)



Fundus (R^2 = 0.0823, p = 0.016073)

Body (R^2 = 0.0004, p = 0.875328)

(b)



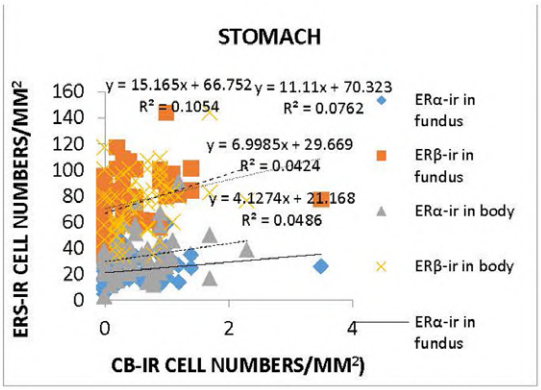
Duodenum (R^2= 0.4923, p = 1.32x10^{-11})

Jejunum(R^2= 0.1773, p = 0.000284)

Ileum(R^2= 0.0635, p = 0.035321335)

Colon(R^2 = 0.1903, p = 0.00016)

(c)



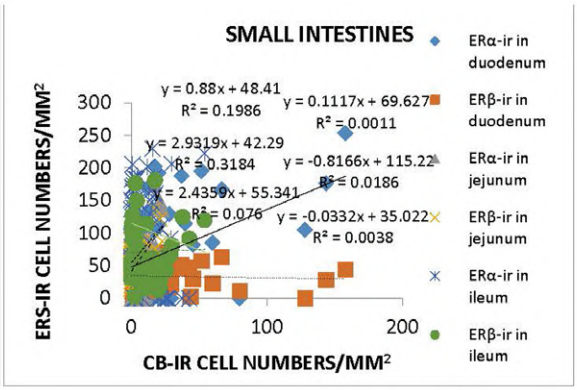
Fundus (CB vs ERα) (R^2 = 0.0486, p = 0.09048)

Fundus (CB vs ERβ) (R^2= 0.0762, p = 0.032729)

Body (CB vs ERα) (R^2 = 0.0424, p = 0.127793)

Body (CB vs ERβ)(R^2= 0.1054, p = 0.014646)

(d)



Duodenum (CB vs ERα)(R^2 = 0.1986, p = 0.000577)

Duodenum (CB vs ERβ) (R^2= 0.0038, p = 0.653447)

Jejunum (CB vs ERα) (R^2 = 0.076, p = 0.039799)

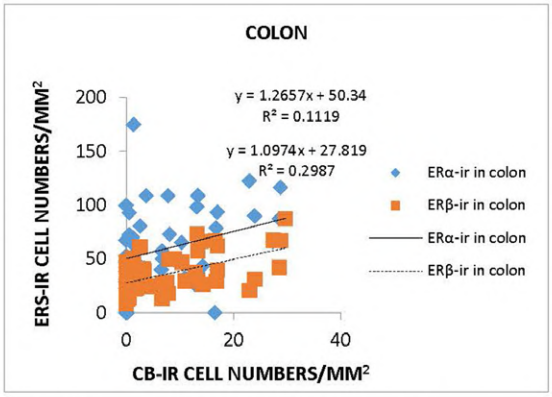
Jejunum (CB vs ERβ) (R^2= 0.3184, p = 5.92 x 10^{-6})

Ileum (CB vs ERα) (R^2 = 0.0186, p = 0.3155753)

Ileum (CB vs ERβ) (R^2= 0.0011, p = 0.80560674)

Fig. 4.- Plot of numbers of the CB-immunoreactive cells against the numbers of ERα-and ERβ-ir cells of the stomach **(a)**, of the small intestines and colon **(b)**; the CB-immunoreactive cells against the numbers of ERα- and ERβ-ir cells of the stomach **(c)**, of the small intestines **(d)** from the same rats and from all animal groups.

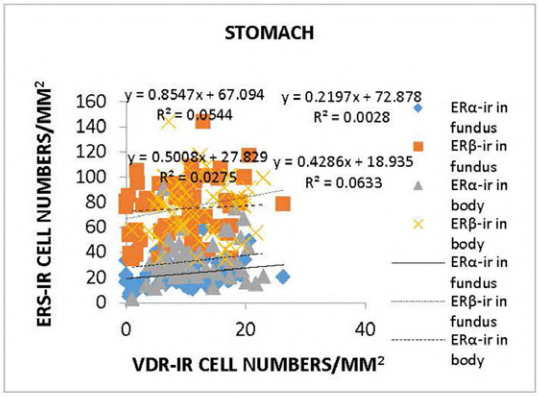
(e)



Colon (CB vs ERα)(R^2 = 0.1119, p = 0.0117366)

Colon (CB vs ERβ)(R^2= 0.2987, p = 1.31 x 10^{-5})

(f)



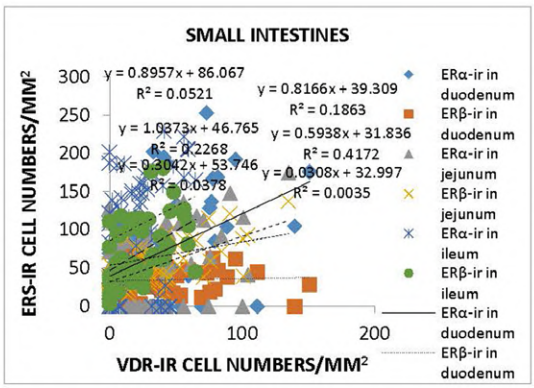
Fundus (VDR vs ERα)(R^2 = 0.0633, p = 0.052576)

Fundus (VDR vs ERβ)(R^2= 0.0544, p = 0.072767)

Body (VDR vs ERα)(R2 = 0.0275, p = 0.221759)

Body (VDR vs ERβ) (R^2= 0.0028, p = 0.698397)

(g)



Duodenum (VDR vs ERα) (R^2 = 0.1863, p = 0.000896)

Duodenum (VDR vs ERβ) (R^2= 0.0035, p = 0.664124)

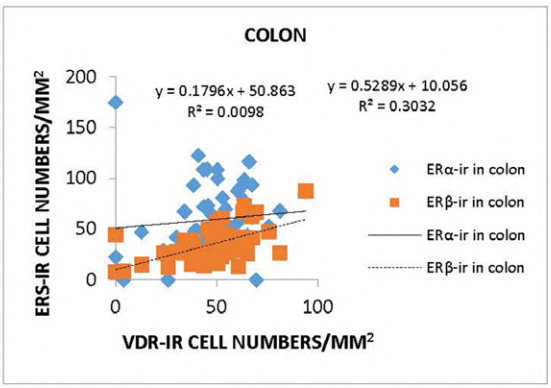
Jejunum (VDR vs ERα) (R^2 = 0.0378, p = 0.150797)

Jejunum (VDR vs ERβ) (R^2= 0.4172, p = 7.62 x 10^{-8})

Ileum (VDR vs ERα) (R^2 = 0.0521, p = 0.090592)

Ileum (VDR vs ERβ)(R^2= 0.2268, p = 0.000207125)

(h)



Colon (VDR vs ERα)(R^2 = 0.0098, p = 0.466974)

Colon (VDR vs ERβ)(R^2= 0.3032, p = 1.097 x 10^{-5})

Fig. 4.- Plot of numbers of the CB-immunoreactive cells against the numbers of ERα-and ERβ-ir cells of the colon **(e)**; the VDR-immunoreactive cells against the numbers of ERα- and ERβ-ir cells of the stomach **(f)**, of the small intestines **(g)**, of the colon **(h)** from the same rats and from all animal groups.

osteoporosis. We were the first research group to report back in 2014 that ORx/sham rats receiving YCJ at 100 mL/kg BW had a significantly higher number of argyrophilic endocrine cells in the GI tract as compared to controls (Radenahmad et al., 2014). Argyrophil endocrine cells are involved in GI functions, e.g., calcium absorption and GI motility, which might influence osteoporosis. These observations, nevertheless, were merely based on special staining, the Gremelius staining used in our study. To take one step further, we conducted the current study to investigate the microscopic changes inside the mucosa, including the epithelium and glands, using an immunohistochemical technique to detect the involvement of calbindin and vitamin D receptors in calcium absorption. Estrogen acts via estrogen receptors (ERs), currently known as ERα and Erβ. Therefore, anti-ERα and anti-ERβ antibodies were used in order to explore the possibility of phytoestrogenic properties of YCJ.

These results confirmed the thesis in our previous study, namely that argyrophilic cells detected by the Gremelius staining and CB-ir, a calcium-binding protein, and VDR-ir cells were mostly restored to normal level after feeding with YCJ in most regions of the GI tract of ORx rats.

The fact that calcitriol exerts its effect on calcium uptake by epithelial cells or other reactions rapidly raises some speculations about the possibility of a second VDR in the plasma membrane, associated with caveolae and a second calbindin protein in the cytoplasm (Norman, 2006). It has been reported that extra-nuclear estrogen receptors associated with the plasma membrane and or cytoplasmic organelles facilitate acute effects of estrogen on neuronal signaling (Hart et al., 2007).

Earlier, calbindin (CaBP-9k) mRNA expression was not detected by Northern blot analysis before in dairy cattle (Yamaguchi et al., 2002). However, a Ca^{2+}-sensing receptor (CaSR) has been identified on the basolateral membrane of rat parietal cells (Caroppo et al., 2001; Cheng et al., 1999). Studies in both rat and human stomachs found that CaSR plays an important regulatory role in acid secretion and mucosal repair (Kirchhoff and Geibel, 2006). We are the first group that identified the presence of calbindin in parietal cells and hypothesized that these cells are involved in calcium absorption. In the stomach, the number of CB-ir cells positively correlated with the numbers of ERα- and ERβ-ir cells, except for CB-ir with ERα-ir in the body of the stomach, implicating the possibility that phytoestrogen of YCJ might have influenced on CB-ir cell production in the stomach. Whether the acid secretion is involved in calcium absorption needs clarification. Nevertheless, the number of CB-ir cells in the stomach was much less than in small and large intestines. This agrees with a report by Lee et al. (2003), using Northern blot that CB-ir cells in the stomach were shown to be much less than that of the duodenum.

The expression of CB was detected primarily in the enterocytes of the duodenal villi. CB functions to regulate the amount of intracellular calcium to prevent cell death from the toxicity of free calcium (Hong and Jeung, 2013). Delorme et al. (1983) reported that the CaBP-9k gene is expressed in rat intestine due to its vitamin D-responsive DNA element (VDRE). However, in humans, this gene is only active in the duodenal mucosa epithelial cells, and its expression progressively decreases downstream from the duodenum, and it is scarcely found in the ileum and large intestine (Walters et al., 1999).

In the present study, very few CB-ir cells were found in the fundus and body of the stomach, and its expression level did not decrease downstream like in humans. The highest number was found in the duodenum, lowest in the jejunum, and increased distally from the ileum toward the colon. However, the highest number was found in the duodenum, as in the previous research reports, e.g., Yamagishi et al. (2002) and Sidler-Lauff et al. (2010). Factors such as the residence time and the absorption rate in a particular intestinal segment determine the amount of calcium absorbed (Wesserman, 1997). This phenomenon could be due to species variation. The number increases once again in the colon, implicating the calcium absorption function of this organ, since it was found that the incidence of osteoporosis increases in IBS (inflammatory bowel diseases) patients (Targownik et al., 2013).

It has been reported that estrogen may play a determinant physiological role in the amount of calcium absorbed from the intestine, where the presence of estrogen receptors has consistently been indicated in the mucosa of the alimentary tract (Fernandez et al., 1996; Francavilla et al., 1987; Hendrickse et al., 1993; Meggouh et al., 1991), which might include post-orchidectomy, as in the present study. The present study found that reduction of testosterone in ORx rats decreased CB production, but EB and YCJ treatments increased its production by cells in many regions of the GI tract, e.g., the fundus of the stomach, the jejunum, and the colon. In this study, YCJ treatment increased the number of CB-ir cells in OJ groups compared with the OE group. This agrees with the report by Patisaul and Jefferson (2010), which stated that soy phytoestrogen has health benefits with fewer adverse effects compared with synthetic (exogenous) estrogen.

Furthermore, the number of CB-ir cells correlated positively with the numbers of ERα- and ERβ-ir cells, except in the ileum. Using Micro-

CT and immunohistochemistry techniques, we found that exogenous estrogen (EB) and YCJ prevent osteoporosis in lumbar (L5) and femur bones (Yooprasert et al., 2015; Asae et al., 2017). Therefore, estrogen/phytoestrogen might influence CB production not only in the stomach but also in small and large intestines that influence bone calcium deposition. Further investigation is needed to clarify whether calcium uptake by parietal cells in the stomach or calcium uptake by enterocytes in the small and large intestine is also regulated by sex steroid hormones, e.g., estrogen, or phytoestrogen, e.g., YCJ, by increasing CB.

Physiologic interactions among various mechanisms of the PTH-vitamin D-endocrine system structured in a multilevel negative feedback loop determine the rate of active intestinal calcium absorption. Active calcium absorption from the intestine occurs in two primary mechanisms. One, through passive diffusion, when the luminal calcium is high, and two, through a more complex active absorption mediated by $1,25(OH)_2D_3$ when the luminal calcium is low (Bringhurst, 1995). Under the stimulation of parathyroid hormone (PTH), the kidney produces $1,25(OH)_2D_3$, where PTH secretion itself is influenced by the extracellular free calcium concentration detected by the calcium-sensing-receptors located in the membranes of parathyroid cells (Chattopadhyay et al., 1996).

With age, there is a decline in intestinal calcium absorption in humans (Avioli et al., 1965; Bullamore et al., 1970) and in rats (Russell et al., 1986). It has been suggested that, besides the PTH and vitamin D, estrogen also has specific physiological functions in the alimentary canal as indicated by the presence of its receptor (Fernandez et al., 1996; Francavilla et al., 1987; Meggouh et al., 1991; Hendrickse et al., 1993), the estrogen-receptor-associated proteins (Theisinger et al., 1993; Luqmani et al., 1992; Welter et al., 1994) and ER-D5 (Takeda et al., 1992) in this organ. Whether this is true with the function of estrogen in the stomach needs further investigation.

We were the first group to discover vitamin D receptors in parietal cells and enterocytes all along small and large intestines. The highest number of VDR-ir cells was found in the duodenum, agreeing with Sidler-Lauff et al. (2010), but in contrast with other reports. Christakos (2011) found that the highest levels of VDR were in the cecum and the colon, implicating the calcium absorption function of this organ and maybe acting together with CB-ir cells. Colonic calcium absorption is vitamin D-responsive, and it becomes important in conditions such as short bowel syndrome (Wada et al., 2009; Wali et al., 1990).

The findings reported by Teerapornpuntakit et al. (2009) and Zhang et al. (2010) reveal that TRPV6 protein and CB are detectable in all intestinal segments, in mice and rats, and the strongest immunoreactivity expression of TRPV6 is in the cecum and the colon. These reports also indicate that, in addition to the duodenum, the intestinal distal segment plays an important function in $1,25(OH)_2D_3$-mediated calcium homeostasis (Christakos et al., 2011).

Following orchidectomy, VDR-ir cells significantly dropped in all six regions examined. The number increased when the ORx rats were treated with EB or YCJ, indicating that estrogen and YCJ administration effectively restored the normal responsiveness of the GI to vitamin D. The finding agrees with reports by Gennari (1990), Civitelli (1988), and Heaney (1978) that estrogen administration was shown to effectively restore the normal responsiveness of the intestine to $1,25(OH)_2D_3$ in ovariectomized premenopausal women (Gennari et al., 1990) and postmenopausal women (Civitelli et al., 1988; Heaney et al., 1978). The number of immunoreactive cells in the OJ groups was higher than that of the OE group, even though the difference was not significant. This phenomenon shows that EB dose injection in this study was sufficient to restore the number of the immunoreactive cells, but phytoestrogen derived of YCJ was more potent than exogenous estrogen (EB) in increasing these cells in almost all regions of the GI tract.

Many reports indicate that CB is not required for vitamin D-dependent intestinal calcium absorption (Perez et al., 2008), and increased intestinal calcium transport has been observed in vitamin D deficient pregnant and lactating rats (Halloron and Deluca 1980; Boass et al.,1981,

Brommage et al., 1990). Krisinger (1991) reported that CB alone could not facilitate 1,25-dihydroxy vitamin D3–mediated increases in intestinal calcium absorption in rats.

In contrast, the results in this study revealed that the number of CB-ir cells positively correlated with VDR-ir in all regions of the GI tract (stomach, small intestine, and colon). Our results agreed with Choi and Jeung (2008), who concluded that intestinal CB is clearly regulated by vitamin D.

A positive correlation (except CB-ir with ERα-cells in the ileum) between CB-ir cells with either ERα- and ERβ-ir may suggest that the active ingredient(s) of YCJ acted through the binding with either ERα or ERβ. It needs to be further investigated if the same cells that were positive for the estrogen receptors were also positive for CB. If co-localization of ERα or ERβ with CB in the same cells exists, it probably means that the expression of CB was due to the binding of YCJ active ingredient(s) with the estrogen receptors. This phenomenon was also found with VDR-ir cells. The number of VDR-ir positively correlated with either ERα- and ERβ-ir cells, suggesting that the active ingredient(s) of YCJ acted through the binding with either ERα or ERβ that caused calcium absorption via CB-ir cells in the "transcellular pathway" along the alimentary tract of ORx rats treated with YCJ.

Estrogenic compounds show different affinities to ERα and ERβ. ERα dominates in several specific tissues in comparison with ERβ that is expressed in other tissues, including bone and the GI tract (Gustafsson, 1999). It is known that endogenous or exogenous estrogens are specific to ERα rather than ERβ. In contrast, containing the phytoestrogen, β-sitosterol, YCJ reacts specifically with ERβ rather than ERα (Kuiper et al., 1998; Sayoh et al., 2008).

It has been indicated by this study that ERβ-ir was found to be much more positively stained than ERα-ir in all six regions of the GI tract examined, particularly in the stomach, where its fundus and body bind stronger to ERβ than ERα. It was reported that both mRNA and enzyme activity of aromatase (P450arom) and estrogen synthetase (Simpson et al., 1994) were detected in the gastric mucosa of rats at levels comparable with that of the ovary, and a high concentration of 17β-estradiol was detected in the portal vein arising from the stomach (Ueyama et al., 2002).

According to Devlin (2011), the two forms of ER have co-expression activity in neurons but different physiological functions, as indicated by the study on knock-out mice. It was reported that ERα is linked to synaptic plasticity, while ERβ to neural cell differentiation. More investigations are required to know the mechanism(s) activated by estrogen/phytoestrogen in the GI tract of male rats.

Researchers hypothesize that a diet rich in isoflavones has a protective effect on the bones (Tham et al., 1998). Even though we found that the YCJ component is primarily beta-sitosterol, since YCJ has a wound-healing effect and almost all isoflavone has a wound-healing effect, phytoestrogen of YCJ could likely be categorized as a member of the isoflavone group. Almost all proteins (antibodies) detected in the present study showed a higher number of immunoreactive cells when ORx rats were treated with lower YCJ treatments (OJ10 and OJ20) than with a higher dose of YCJ treatment (OJ40). Vincent and Fitzpatrick (2000) found that genistein, a kind of isoflavone in ovariectomized rats, has a biphasic effect. Lower doses improved bone mineral as opposed to high doses on bone mineral density. Therefore, YCJ could act as a kind of selective estrogen receptor moderator.

The possibility of the skeletal effects of testosterone is believed to be mediated by the aromatization of androgen to estrogens, as confirmed by findings of the presence of osteoporosis in men with mutations in aromatase gene (Carani et al., 1997) or estrogen receptor gene (Smith et al., 1994). Furthermore, Khosla et al. (1998), Slemenda et al. (1997), and Darko Kastelan et al. (2006) reported that estrogen rather than testosterone levels are more closely correlated with bone mineral density in older adults.

CONCLUSION

We were the first research team that found that there were CB-ir and VDR-ir in parietal cells of the stomach and the enterocytes of intestinal villi

of the small intestine and the colonocytes of the colon. The results of this study revealed that: (1) EB injection at 2.5 µg/kg BW/day for three days per week is enough to restore all immunoreactive cells detected by anti-CB-, anti-VDR-, anti-ERα- and anti-ERβ-ir antibodies. (2) The various doses of YCJ treatment restored the decreased numbers of CB-, VDR-, ERα- and ERβ-ir caused by orchidectomy to normal or close-to-normal levels; (3) the effects of YCJ were comparable to those of EB treatment. In most cases, the doses of YCJ at 10 mL/kg BW/d were the best. (4) This study indicates that not only CB and VDR but also estrogen/phytoestrogen is important in calcium absorption involved in the maintenance of calcium homeostasis. These findings indicate that feeding YCJ could account for, at least in part, the protective role of estrogen replacement against osteoporosis in older adults or men with hypogonadism.

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