Young coconut juice significantly reduces histopathological changes in the brain that is induced by hormonal imbalance: A possible implication to postmenopausal women

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Summary. Background and aim: Some degenerative diseases of the nervous system have been linked to hormonal imbalance in postmenopausal women. It is argued that young coconut juice (YCJ) could have some estrogen-like characteristics, but this is still debatable. Our aim was to investigate this argument, and to examine whether YCJ has any neuroprotective effects.

Materials and methods: Four groups of female rats (10 in each group) were included in this study. These included sham-operated, ovariectomized (ovx), ovx and receiving estradiol benzoate (EB) injections intraperitoneally, and ovx and receiving YCJ orally. At the end of the five-week study, the rats were sacrificed, and their serum estradiol (E2) level was measured by chemiluminescent immunoassay. Moreover, the rat brains were excised, and the cortical pyramidal neurons were examined using markers of neuronal cell death, namely anti-neurofilament (NF200) and anti-parvalbumin (PV) antibodies.

Results: Our results showed that the rat group which received YCJ had its serum E2 level significantly (P<0.05) higher than the ovx group which did not receive any treatment, and the sham-operated group. A similar trend was observed with the group which received EB injections, but no significant difference was present when the latter was compared with the sham-operated group. In addition, a significant reduction in neuronal cell death was observed in the YCJ-treated group, as compared to the ovx group which did not receive any treatment. This was indicated by the significantly (P<0.05) higher number of neurons which were immunopositive for NF200 and PV. Interestingly, the number of these neurons was also significantly (P<0.05) higher in the YCJ group, as compared to the EB group.

Conclusion: This study confirms the argument that YCJ has estrogen-like characteristics, and it also adds more evidence to the observation that hormonal imbalance could induce some brain pathologies in females.

Key words: Young coconut juice, Neuron cell death, Estrogen, Neurofilaments, Parvalbumin, Menopause, Ovariectomy

Introduction

Phytoestrogens are important components of human nutrition. They include isoflavones, which have been found to have a strong agonistic effect on the estrogen receptor-beta (ER-β), and a weaker one on ER-α (Akiyama et al., 1987; Feller and Wong, 1992; Peterson, 1995). They also include genistein, which was found to be a potent inhibitor of protein tyrosine kinase (Akiyama et al., 1987; Feller and Wong, 1992; Peterson, 1995). The latter seems to play a key role in growth factor-related-signal modulation, as well as in programmed cell death or apoptosis (Akiyama et al., 1987; Feller and Wong, 1992; Peterson, 1995).
Exogenous estrogen can reduce the risk of dementia in postmenopausal women. In particular, 17ß-estradiol seems to exert a wide variety of actions in the brain, affecting behavior, cognition, and cell viability (McEwen and Alves, 1999). Estrogen has been found to promote plastic changes in dendritic spines, and to increase synaptic density in specific brain regions of the rat, including the hippocampus (McEwen, 1996; Wooley, 1998; Choi et al., 2003). Animal studies and in vitro experiments have demonstrated that estradiol can prevent neuronal cell death often induced by excitotoxic insults, such as amyloid-ß neurotoxicity, and can ameliorate some of the effects of aging, such as hippocampal degeneration and cognitive impairment (Weaver et al., 1997; Green et al., 2000; Wise et al., 2001; Petanceska et al., 2000; Foster et al., 2003).

Pyramidal cells in layers III and V are cortical neuronal projections, while non-pyramidal cells are inter-neurons that are mostly subpopulations of GABAnergic neurons. Depletion of either pyramidal or non-pyramidal neurons often results in neuronal circuitry interruption, neuronal degeneration, and brain dysfunction. Several investigations have reported the presence of a relationship between steroid hormones and neurotransmitters (Gould et al., 1990; Wooley and McEwen, 1993; McEwen et al., 1999). Estrogen induces structural and functional changes in excitatory inputs to the hippocampal CA1 pyramidal cells in adult female rats. Furthermore, estrogen increases the density of dendritic spines, as well as spine synapses in CA1 pyramidal cells, concomitant with an increase in peak Ca2+ levels (Gould et al., 1990; Wooley and McEwen, 1993; McEwen et al., 1999).

Ca2+ binding proteins have been proven to be useful neuronal markers for a variety of functional brain systems and their circuits. Their major roles are assumed to be Ca2+ buffering, transport, and regulation of various enzyme systems. Since cellular degeneration is accompanied by impaired Ca2+ homeostasis, a protective role for Ca2+ binding proteins in certain neuron populations has been postulated. These proteins include neurofilament-related (NF200) proteins, and parvalbumin (PV). As neuronal degenerative changes occur in several brain diseases in humans, such as Alzheimer’s disease, Parkinson’s disease, and epilepsy, changes in the expression of Ca2+ binding proteins have been studied during the course of these diseases, and have been found to alter based on the progress of the disease (Heizmann and Braun, 1992). Estrogen level seems to affect these proteins by modulating signaling in a calcium-dose dependent manner (Rajadhyaksha et al., 1998). PV is a calcium-binding protein. It participates in the regulation of calcium homeostasis, and acts as an intracellular calcium buffer regulating the concentration of the ion during neuronal activity (Kawaguchi et al., 1987). PV has been reported to be associated with specific subpopulations of GABAnergic neurons in the cerebral cortex and hippocampus, and, along with neurofilaments, it has been used as an index of neuronal cell death, as reported in degenerative brain diseases like Alzheimer’s (Celio, 1986; Kosaka et al., 1987; Greene et al., 2001; Radenahmad et al., 2003).

According to folk medicine, coconut juice (Cocos nucifera L., Arecaceae) is claimed to contain several different compounds with various therapeutic properties. In Tropical Asia, especially in Southeast Asia, women were prohibited from drinking coconut juice because it disrupted their natural menstrual cycle. Accordingly, folk medicine postulated that young coconut juice (YCJ) has phytoestrogen-like effect. This is confirmed by our recent results, where some brain pathologies were significantly-reduced in the brains of overiectomized (ovx) rats which received YCJ, as compared to overiectomized rats which did not receive any treatment (Radenahmad et al., 2006). Phytoestrogens seem to have other beneficial effects, and flavonoids like kaemferol, isoflavones, luteolin, and apigenin seem to be among their essential components (Spilkova and Hubik, 1992; Havsteen, 2002).

In this study, we investigated the possible neuroprotective effects of YCJ by examining its ability to halt neuronal cell death in ovx rats, a model used for postmenopausal women. For this purpose, we used anti-neurofilament and anti-parvalbumin (NF200 and PV) antibodies to assess the cytoskeletal and calcium-binding protein (CaBP) changes, respectively, in the cortices of the parietal, temporal, and occipital lobes.

Materials and methods

Plant material

YCJ (Cocos nucifera L., Arecaceae) was collected from Namnoi district, Hat Yai, Songkhla, Thailand. It was then processed by Associate Professor Sanan Subhadhirasakul at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Thailand. YCJ was dried, and the powder formed was kept at -30°C until used. This powder was freshly reconstituted and prepared for oral intake every day. The complete description of YCJ, including its preparation and administration, is provided in our previous publication (Radenahmad et al., 2006).

Animals

All animals used were adult four-month female Wistar rats weighing approximately 270 g. The animals were housed in a controlled environment at 25±1°C on an illumination schedule of 12hrs light/12hrs dark cycle. Rats had unrestricted access to standard pellet food and water. The study was approved by the 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.

Experimental design

There were four groups (10 rats per group) included
in this study. The first group consisted of ovx rats, the second group consisted of sham-operated rats, the third group consisted of ovx rats injected intraperitoneally with exogenous estrogen (2.5 g/kgBwt of estradiol benzoate, EB) twice a week for four weeks, and the fourth group consisted of ovx rats which received YCJ (100 ml/kgBwt/day) for four weeks. The dose of EB and YCJ in this study was based on the one reported in our earlier study and in which dose standardization and optimal administration were set (Radenahmad et al., 2006). In this study, the administration of EB and YCJ was started one week after ovariectomy was performed. Rats belonging to the first and second groups received Milli Q water instead.

**Immunohistochemistry**

Tissue samples from the cerebral cortex were processed into paraffin blocks, and ten 4µm sections were collected from each block and mounted on 3-aminopropyltriethoxysilane (TESPA; Sigma)-coated slides. Anatomical orientation to the rat brain was facilitated by using a rat brain anatomy atlas (Paxinos and Watson, 1986). The first two sections were stained with cresyl violet, and were used for light microscopy examination and anatomical orientation. Three of the remaining eight sections were immunostained with the anti-NF200 monoclonal antibody (N-0142, Sigma, USA), another three sections were immunostained with the anti-PV monoclonal antibody (P3088, Sigma, USA), while the remaining two sections were used as negative controls. The immunostaining technique was as follows. Sections were pre-incubated at 52°C for 2hrs prior to commencing staining. This was followed by passing the sections through serial steps of fresh xylene and alcohol gradients. The sections were then left in distilled water for 10 minutes before antigen retrieval by microwave for half an hour was performed (0.01% sodium citrate gradients). The sections were then allowed to cool down at room temperature for 15 minutes, following by washing with TBS, and incubation for 10 minutes with 0.3% H2O2 in methanol to quench minutes, followed by washing with TBS, and incubation while the remaining two sections were used as negative. The immunostaining technique was as follows. Sections were pre-incubated at 52°C for 2hrs prior to commencing staining. This was followed by passing the sections through serial steps of fresh xylene and alcohol gradients. The sections were then left in distilled water for 10 minutes before antigen retrieval by microwave for half an hour was performed (0.01% sodium citrate antigen retrieval solution; 650 Watts). The sections were then allowed to cool down at room temperature for 15 minutes, followed by washing with TBS, and incubation for 10 minutes with 0.3% H2O2 in methanol to quench any endogenous peroxidase activity. They were then incubated with the primary antibodies (anti-NF200, 1:100, and anti-PV, 1:100) for 30 minutes in a humidified chamber, washed with TBS, and then incubated with the secondary and tertiary antibodies with frequent TBS washes in between. The procedure was followed according to the steps provided with the ABC kit used (Vectastain® Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). The substrate chromogen (one minute incubation time) used in this study was diaminobenzidine (DAB) enhanced by the addition of a nickel solution from the staining kit (Vector Laboratories, Burlingame, CA, USA). Sections were then counterstained with Meyer's hematoxin for five seconds, and cover-slipped. Negative controls included sections in which incubation with the primary antibody was replaced by TBS. Positive controls were cortical brain sections known to be positive for NF200 and PV.

**Quantitative analysis of immunoreactive cells**

Counting of the cortical neurons positive for NF200 and PV was performed by two independent observers using an image analysis system (IAS). The IAS consisted of an observer-interactive computerized image analysis (SAMBA microscopic image processor; Meylan, France). This system is fitted with a standard aixoan microscope with an automated stage (Carl Zeiss; Oberkochen, Germany) allowing a precise location of a particular field through the XYZ axis plotting, a colour video camera (Sony Corporation; Tokyo, Japan), an image analysis processor (Matrox; Montreal, QC, Canada), and a personal computer (Pentium 2, 166-MHZ processor; Intel; Santa Clara, CA). Counting was done in ten random fields at x400 magnification. Readings from both observers were then added and the average was determined. Random selection of the fields was achieved using a computer generated list of random numbers (Excel version 5.0). Data was expressed as number of neurons per µm². The mean ± SEM was used to compare the four groups.

**Serum estradiol**

All the rats were sacrificed on the first day of the sixth week. Their serum was collected through a carotid catheter for estradiol (E2) measurement using the chemiluminescent immunoassay (CIA) technique, as set by the manufacturer (CIA kit LKE2 10261, DPC, Gwynedd, UK). The steps followed were the following:

First incubation: we incubated the sample with an estradiol-specific biotinylated antibody, which resulted in the formation of an immunocomplex.

Second incubation: Following the addition of streptavidin-coated microparticles and an estradiol derivative labeled with a ruthenium complex, the still-vacant sites of the biotinylated antibodies became occupied, with formation of an antibody-hapten complex. The entire complex became bound to the solid phase via interaction of biotin and streptavidin.

The reaction mixture was then aspirated into the measuring cell, where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were removed with ProCell. By applying a voltage to the electrode, we induced chemiluminescent emission which was measured by a photomultiplier. The measuring range is 5.00-4300 pg/mL.

The results were then determined via a calibration curve which was instrument-specifically generated by 2-point calibration, and a master curve provided via the reagent barcode.

For quality control, we used the Elecsys PreciControl Universal 1 and 2. This was performed every time we run the test on our samples.

The CIA technique which we used has been reported...
to have high sensitivity and specificity, whereby no interference has been documented thus affecting the actual readings (Igbal et al., 1983; Lichtenberg et al., 1992; Tietz, 1995).

**Statistical analysis**

Statistical analysis was performed using the Kruskal-Wallis and the Mann-Whitney U-tests available in the statistical program SPSS version 13.0. 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 mean SEM. P<0.05 was considered significant.

**Results**

**Serum E2 level**

Our results showed that the rat group which received YCJ had their serum E2 level significantly (P<0.05) higher than the overiectomized group (Fig. 1). A significant difference (P<0.05) was also observed when compared with the sham-operated group. It was interesting to notice that YCJ was capable of bringing E2 to a level even higher than the group receiving EB.

**Immunohistochemistry**

Associated with the significant restoration of the serum E2 level following the oral intake of YCJ, a significant reduction in neuronal cell death was observed in this animal group as compared to the overiectomized group which did not receive any treatment (Figs. 2, 3). This was indicated by the significantly (P<0.05) higher number of the neurons which were positive for NF200, and PV. Interestingly, the number of these neurons was also significantly (P<0.05) higher in the YCJ group, as compared to the EB group.

**Discussion**

There is an increasing volume of evidence to support the idea of using phytoestrogens as an alternative strategy to hormone replacement therapy (HRT) since they can also produce other general health benefits. These include a reduction in the risk of HRT-induced cancers, and the prevention of osteoporosis and cardiovascular diseases. None of these suggestions has previously been seriously tested, especially the use of YCJ, to prevent cognitive decline. It has been established that higher cortical functions, such as behavior and memory, could be assessed by examining the degree of expression of the cerebral cortex of NF200 and PV (Iuvone et al., 1996; Hwang et al., 2006). The purpose of this study was to determine whether YCJ, which has been previously found to contain estrogen-like compounds (Punghmatharith, 1988), had any beneficial effect on preserving neuronal cells in ovx rats.

SMI32 is a mouse monoclonal antibody initially developed by Sternberger and Sternberger (Sternberger and Sternberger, 1983). It is directed against the carboxy terminus of the non-phosphorylated form of the medium and heavy neurofilament proteins, and it has been used to identify these proteins in primate and human brains (Hof et al., 1995). These proteins have been found to be associated with Alzheimer’s and other neurodegenerative diseases (Morrison et al., 1987; Hof et al., 1990; Law and Harrison, 2003). Previously, some members in our group were capable of examining the pattern of neuronal staining using another mouse monoclonal antibody directed against the NF-H protein, which even recognized both the phosphorylated and non-phosphorylated epitopes (Radenahmad et al., 2003). In this study, we used the same antibody (NF200) to examine the benefit of oral intake of YCJ on the brain cortical neurons in ovx rats.

The relation between the ovaries and brain neurons is a field which has drawn attention recently, and it is based on the following observations. The ovaries are the main site of production of the estrogen hormone. It has been reported that estradiol can enhance plasticity and survival of the injured brain, and that estradiol levels could protect against neuronal degeneration (Dubal et al., 1999, 2001, 2006; Shughrue and Merchenthaler, 2000; Merchenthaler and Shughrue, 2005). This is achieved through alterations in bcl-2 gene expression, as well as through actions involving the estrogen receptors ER-α and ER-β (Dubal et al., 1999, 2001, 2006; Shughrue and Merchenthaler, 2000; Merchenthaler and Shughrue, 2005).

The results obtained in this study showed that the oral intake of YCJ significantly reduced the number of degenerating cortical neuronal cells, which was induced by overiectomy. This was demonstrated by the significantly higher number of neurons which were positive for NF200. The majority of these pyramidal cells were in the deep part of lamina V, with a smaller number in lamina III. A similar trend was observed with PV. The significant reduction in the cortical neurons

**Fig. 1.** Serum estradiol (E2) level (mean ±SEM) in the four groups examined. ovx: overiectomized group; ovx + EB: overiectomized group receiving estradiol benzoate; ovx + YCJ: overiectomized group receiving young coconut juice. n=10 in each group. *P<0.05.
Young coconut juice and hormonally-induced histopathological changes in the brain

Fig. 2. A. Number of pyramidal neurons positive for the neurofilament antibody NF200 in the occipital, parietal, and temporal cortical brain regions in the four rat groups. ovx: ovariectomized group; ovx + EB: ovariectomized group receiving estradiol benzoate; ovx + YCJ: ovariectomized group receiving young coconut juice. n=10 rats in each group. *P<0.05.

B and D. Neurons strongly positive for NF200 in the cortical brain regions of the rats in the sham group, and in the ovx group which received intraperitoneal injections of EB, respectively.

C. Higher magnification of the red box field in B. E and F. Note the weak or negative cell body staining, as well as the lack of the preservation of these bodies in the ovx group. G. Note the strong positive immunostaining, as well as the good preservation of the cell bodies in the ovx group receiving YCJ.
Young coconut juice and hormonally-induced histopathological changes in the brain

Fig. 3. A. Number of pyramidal neurons positive for the Parvalbumin (PV) monoclonal antibody in the occipital, parietal, and temporal cortical brain regions in the four rat groups. ovx: overiectomized group; ovx + EB: overiectomized group receiving estradiol benzoate; ovx + YCJ: overiectomized group receiving young coconut juice. n=10 rats in each group. *P<0.05.

B. Strong PV-positive staining of pyramidal cortical neurons in the brains of the sham-operated rat group.

C. Higher magnification of the red box field in B. D and E. Strong PV-positive staining of pyramidal cortical neurons in the brains of the ovx group which received YCJ. F. Very few neurons showing positive staining for PV, as compared to many negative ones (orange arrows) in the brains of ovx rats. G. Strong PV-positive staining of pyramidal cortical neurons in the brains of the ovx group which received intraperitoneal injections of EB.
positive for NF200 and PV in the cerebral cortices of the ovx group, and the restoration of such reduction following administration of exogenous estrogen by EB injection or oral intake of YCJ indicates that estrogen could have a significant effect on the cytoskeleton and calcium homeostasis of the above neurons. Interestingly, such restoration was better displayed in the group receiving YCJ, as compared to the group receiving EB. This indicates that YCJ has strong estrogen–like properties. It is possible that the receptors for the estrogen-like components of YCJ have greater affinity, as compared to those of EB. Kuiper and colleagues found that phytoestrogens exhibit a great affinity for ER-β. This finding is of interest especially in that the expression level of ER-β has been found to be high in the brain regions critical to memory function and vulnerable to Alzheimer’s and other degenerative diseases (Vickers et al., 1994; DeFelipe, 1997; Kuiper et al., 1998; Stahl et al., 1998; Liu et al., 2003).

Using radioimmunoassay techniques, Punghmatharith found that 1 ml of coconut juice contained 2.45 pg of 17β-estradiol, in addition to other sex hormone-like substances like estrone-3-glucuronide, pregnanediol-3-glucuronide, progesterone, testosterone, and estrone (Punghmatharith, 1988). Results from thin-layer chromatography studies also confirmed that YCJ contained substances similar to estrone, 17β-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/kg BW/day for 3 consecutive days significantly increased the uterine wet weight of immature rats. Furthermore, YCJ has been found to contain β-sitosterol (58%), as well as other sterols like stigmastatrienol, stigmasterol, fucosterol, α-spinasterol, etc. β-sitosterol is structurally related to animal cholesterol, and could possibly act as a precursor of sex steroids (Moghadasian, 2000; Radenahmad, 2000). In the present study, β-sitosterol and stigmasterol (plant sterols known to synthesize steroid hormones in vivo) could be responsible for the estrogenic effects of the YCJ. This remains to be confirmed by our group in subsequent studies.

In conclusion, this study provides further evidence that YCJ has neuroprotective effects in conditions that mimic menopause in humans. It also supports our recent work, whereby the oral intake of YCJ was found to decrease the percentage of Aβ1-42, Tau 1, and astrogliosis-induced pathologies in the brains of ovx rats (In preparation). Moreover, ovx rats could be useful models for evaluating the impact of natural estrogen derivatives and estrogen–like compounds on neurodegenerative biomarkers in vivo. The exact mechanism(s) of action of YCJ, as well as its integration in clinical human trials remain to be investigated and achieved. Also, the current average daily consumption of Asians for YCJ is one coconut fruit a day (about 400-500 ml of YCJ per coconut). Whether such a daily consumption is sufficient to prevent neuronal degeneration in people who are prone to developing Alzheimer’s disease remains to be studied.

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Young coconut juice and hormonally-induced histopathological changes in the brain

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