Article

Beneficial Effects of Young Coconut Juice on Increasing Skin Thickness, Enhancing Skin Whitening, and Reducing Skin Wrinkles in Ovariectomized Rats

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Abstract: It has been previously demonstrated in light microscopic and immunohistochemical studies that ovariectomized rats receiving young coconut juice at 100 mL/kg BW showed much better wound healing and improved skin complexion. Nevertheless, it was found that young coconut juice at 100 mL/kg BW/day caused unfavorable side effects, such as glycogen deposition in the liver. Therefore, in the current study, 3 lower doses (10, 20, and 40 mL/kg BW/day) were optimized, and the ultrastructure was further investigated. Compared to normal rats, all the parameters regarding skin changes, including epidermal and dermal thickness, the number of hair follicles, the diameters of collagen fibrils, perimeters, and nuclei of fibroblast and keratinocyte cells, and ultrastructural changes in keratinocyte and fibroblast cells were significantly reduced in ovariectomized rats. Those parameters in the ovariectomized rats were restored to normal by injecting estradiol benzoate or by feeding young coconut juice to the rats, where the effect was found to be dose-related but not in the case of all the parameters. In most cases, a dose of young coconut juice of 40 mL/kg BW/day was the optimal dose. The results suggest that young coconut juice may be as effective as estradiol benzoate in reducing skin atrophy/aging, probably as a selective estrogen receptor moderator.

Keywords: young coconut juice; skin; keratinocytes; fibroblasts; transmission electron microscopy (TEM)

1. Introduction

The skin is the body’s biggest organ. As it gets older, it starts to degenerate. The skin serves a variety of purposes, including protecting the body, regulating temperature, and acting as a sensory organ [1]. Skin aging is characterized by a loss of elasticity, decreased epidermal thickness, and the degeneration of dermal collagen and elastic fibers, resulting in wrinkles and skin dryness [2].

Hormones have an affinity with the skin. Estrogens influence skin’s water-holding capacity [3], vascularity [4], pigmentation [5], and elasticity [6], among other skin functions. Estrogens influence skin thickness, wrinkles, and hydration, thus helping to prevent skin aging [7]. Estrogens influence the skin and its appendages, such as hair follicles [8]. Ovarian estrogen is a major source of estrogen in women, but it decreases with age, especially following menopause [9]. The skin of menopausal women undergoes degenerative changes. The skin’s thickness declines by 1.13 percent every year after menopause, whereas the collagen content decreases by 2 percent [10]. The skin’s ability to protect itself against oxidative stress is also impaired by estrogen deficiency. Collagen deficit causes the skin to become thin, losing suppleness and demonstrating wrinkles, dryness, and vascularity [2].
Estrogen replacement therapy can improve keratinocyte proliferation, epidermal thickness, skin suppleness, collagen content and quality, and vascularization, and reduce wrinkles, all of which effects can help to reverse these skin changes [8,11,12]. Hormone replacement therapy (HRT) can help protect the skin as one becomes older, especially if one is going through menopause (the period when estrogen levels drop dramatically). Despite this, HRT increases the risk of a variety of cancers, including invasive breast cancer [13] and uterine cancer [14,15], as well as cardiovascular illness, including an increased risk of heart attack, stroke, and blood clots [13]. Phytoestrogens are naturally occurring selective estrogen receptor modulators (SERMs) that can provide a natural estrogen replacement for postmenopausal women.

This research team discovered in 2006 that ovariectomized (ovx) rats given young coconut juice (YCJ) demonstrated considerably improved wound healing (including reduced scarring), brighter skin, and shorter hair than controls [16]. The phytoestrogenic properties of YCJ [17], binding with ERα and ERβ [18] and downregulating the macrophage migratory inhibitory factor (MIF) [19], were later discovered to be responsible for the favorable effects of YCJ on accelerating cutaneous wound healing. At 100 mL/kg BW/day, the YCJ supplement had adverse side effects, such as glycogen accumulation in the liver [18]. As a result, three lower dosages of YCJ (10, 20, and 40 mL/kg body weight, respectively) were used in the current investigation. Using ovariectomized rats as a model for postmenopausal women, the current study aimed to determine the optimal dose of YCJ, with the fewest adverse effects on cutaneous alterations, to alleviate skin problems in aging/menopausal women using light microscopy (LM) and transmission electron microscopy (TEM).

2. Materials and Methods

2.1. YCJ Preparation

YCJ was collected in considerable quantities in the Khlong Hoi Khong region of Hat Yai, Songkhla, Thailand. It was then dried, and the resulting powder was stored at −30 °C until it was needed. The powder was newly reconstituted and prepared for oral consumption daily. Our earlier paper [16] contains a detailed explanation of the YCJ preparation process and administration protocol.

2.2. Animals

Mahidol University’s Salaya campus provided adult female Wistar rats (8 months old and 250–300 g BW). At the Animal House Laboratory, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand, the animals were fed standard food pellets, housed in a room free of any sources of chemical contamination, artificially illuminated (a 12-h dark/light cycle), and exposed to controlled temperatures (25 ± 1 °C) and humidity (50 ± 5%). The Animal Care and Use Committee of Prince of Songkla University and the National Institute of Health (NIH publication 86–23, revised 1985) provided humane treatment for all animals. License number 04/57 was assigned to the protocol.

2.3. Experimental Design

Seventy rats were randomly allocated into seven groups, each containing ten rats. The first group served as a standard control group (NC). The second group is the sham-operated group (SC). The ovariectomized (ovx) rats were the third group (OC). Exogenous estrogen (2.5 g/kg BW) was injected into the fourth group of ovx rats, and the assigned dose of estradiol benzoate was given for three days a week (OE), following the same procedure as in earlier investigations [20]. The ovx rats in the fifth, sixth, and seventh groups were given 10 (OJ10), 20 (OJ20), and 40 (OJ40) mL/kg BW/day of YCJ, respectively. One week following the ovariectomy, the EB and YCJ treatments began. Here, an injection vehicle (reverse osmosis water) was force-fed to SC and OC rats. One week after the ovariectomy, YCJ was treated once a day, every day. The rats were sacrificed after ten weeks of feeding and injection treatments. For the LM and TEM studies, the skin from the dorsal region of each animal was excised, fixed with 10% neutral formalin, and processed with paraffin. The
chemiluminescent immunoassay (CIA) technique was used to obtain serum for estradiol assays (ECLIA, Modular E 170C, Estradiol II 03,000,079 122, Roche, Germany).

Ovariectomy Procedure

The lateral side of the rats was cleaned with antiseptic solutions. The animal was anesthetized then placed on the operating table, with the lateral side of the body in an upright position. A single midline incision was made about one fingerbreadth below the costal margin, and the skin was penetrated using small surgical scissors. Blunt forceps were used to free subcutaneous connective tissue from the underlying muscle of each side. The ovary was located by retracting the surrounding fat around the ovary to expose the oviduct. All vessels around the ovary were ligated to avoid bleeding before removing the ovary out of the fat pad. For the sham group, after the operation, the ovaries were put back into the abdominal cavity, then the subcutaneous tissue, muscle layer, and, finally, the skin were sutured.

2.4. Specimen Preparation for TEM

Tissue samples from the rats’ dorsal skin were fixed for 24 h at 4 °C in 4% paraformaldehyde (primary fixation), then in 1% OsO4 for 1 h, washed with phosphate buffer solution, then blocked with 2% uranyl acetate for 1 h. The specimens were dehydrated using increasing quantities of ethanol, before being embedded in Embed-812 (Epon-812 Substitute, Cat # 14900). To assess the tissue area, semi-thin slices were cut at 400 nm and stained with 0.5% toluidine blue. Before being examined with a transmission electron microscope, ultrathin sections were cut at 90 nm and stained with 5% uranyl acetate and lead citrate (JEOL JEM-2010, Tokyo, Japan).

2.5. Microscopic Analysis by Quantitative Histomorphometry

2.5.1. Light Microscopy: Hematoxylin and Eosin (H&E) Staining

Hematoxylin and Eosin Staining

Briefly, the sections were deparaffinized using xylene. Then, the sections were hydrated using grading alcohol, then washed with tap water. Primary staining of the nuclei was conducted with Mayer’s hematoxylin and rewashed with tap water. Eosin was used to counterstain these sections, and they were dehydrated with grading alcohol, cleared with xylene, and finally cover-slipped with permount.

Measurement of Epidermal and Dermal Thickness; the Number of Hair Follicles

Two impartial observers counted the parameters being studied. At 400× magnification, an eyepiece micrometer was mounted on a light microscope, and a count was taken. The average was calculated by adding the readings from both observers. The distance between the stratum granulosum and the stratum basale was used to determine epidermal thickness. Dermal thickness was determined by measuring the distance between the stratum basale and the upper section of the hypodermis. The distance between the dermis’ bottom line and the muscular adipose was used to calculate hypodermal thickness.

Each thickness measurement is based on an average of 25 measurements. Hair follicles with a diameter of more than 20 µm were detected in ten regions of the dermis, averaged, and compared between groups. Image analysis software was used to analyze and quantify all parameters (Olympus Cellsens version 1.6, Tokyo, Japan). The results were represented as numbers per µm², and the seven groups were compared using the mean ± SEM.

2.5.2. Transmission Electron Microscopy (TEM)

- For the measurement of collagen fibril diameters, for 1 grid, collagen fibrils were photographed at 20,000× magnification in 2 planes: long- and cross-sections. For a long section, 2 photos were taken, and 20 collagen fibrils per photo were measured and averaged. For a cross-section, 60 collagen fibrils were measured and averaged. Therefore, for one grid, a total of 100 fibrils were counted, and the results were
Averaged [21]. A statistical comparison of the seven groups was made using each group’s mean ± SEM data.

In measuring cellular changes, ten cells from one grid were randomly studied at 4000× magnification, using the criteria employed by Hussein et al. [22].

- Measurement of the nucleus and cell perimeter

The ultrastructure (TEM) photos of each rat’s ten largest keratinocytes and fibroblasts (3 rats per group) were chosen. They were then transferred to the microscope program, and the nucleus and cell perimeters were drawn and measured. Two impartial observers counted the parameters, and the average values of the observation results were determined. Readings from both the observers were added, and an average was determined. Image analysis software (Olympus BX50 light microscope and Olympus DP73 camera with cellSens software) was used to analyze and quantify all parameters (v1.16, Olympus Corporation). The results were reported as nucleus/cell perimeters per µm, and the seven groups were compared using mean ± SEM.

2.6. Statistical Analysis

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

3. Results

3.1. LM (Hematoxylin and Eosin Staining): Epidermis and Dermis

The epidermis and dermis of skin changes stained with Hematoxylin and Eosin staining are demonstrated in Figure 1. Following ovariectomy (OC group), the skin of the ovx rats showed typical atrophy. The degree of keratinization and thickness of the epidermis showed a reduction.

![Figure 1. Upper row. A comparison of rat skin epidermal thicknesses for the 7 groups examined (Hematoxylin and Eosin staining, 40×). The epidermal thickness of the OC group was the thinnest, while that of the OJ10 group was the thickest among 3 doses of YCJ treatment. Yellow arrows indicate the epidermal layers. Bar = 50 µm. Lower row: A comparison of rat skin dermal thickness for the 7 groups examined (H&E, 10×). Dermal thickness for the OC group was the thinnest. Among 3 doses of YCJ treatment, the OJ40 group was the thickest. The number of hair follicles in the dermis of the OJ40 group was the most abundant. Yellow arrows indicate the dermal layers. Bar = 200 µm. NC = the baseline normal control group; SC = sham-operated group; OC = ovariectomized (ovx) group; OE = ovx rats receiving estradiol benzoate 2.5 µg/kg BW/day; OJ10 = ovx rats receiving YCJ at 10 mL/kg BW/day; OJ40 = ovx rats receiving YCJ at 40 mL/kg BW/day.](image-url)
The epidermal thickness became thinner in comparison with the control groups; the keratinocyte cell number and the vascularity were low; the number of glands was reduced; the collagen bundles’ morphology and distribution changed. EB injections (OE group) or YCJ injections (OJ group) reduced the histological changes in the skin caused by the ovariectomy (OC group). In fact, the epidermal, as well as dermal, keratinization and thickness in ovx-treated rats were virtually unchanged compared to control animals (SC and OE groups). When ovx rats were given EB (OE group) or YCJ treatments, their epidermal and dermal thicknesses increased (OJ groups). Skin thickness was enhanced with YCJ treatments (OJ10, OJ20, and OJ40 groups), but not in a dose-dependent way. The OJ10 group had the highest epidermal thickness (Figure 2A), while the OJ40 group had the highest dermal thickness (Figure 2B). Furthermore, the morphology and distribution of the collagen bundle of the YCJ treatment groups were equivalent to that of the control group (NC, SC, and OE groups). When the number of hair follicles and the diameter of collagen fibrils were measured, the OJ40 groups had the highest scores (Figure 2C–E).

![Skin parameters](image)

**Figure 2.** Skin parameters: Epidermal thickness (A), dermal thickness (B), numbers of hair follicles (C), collagen fibril diameter from thick sections (D), collagen fibril diameter from thin sections (E). Significant differences at $p < 0.05$ were shown by the different letters indicated above the columns. NC = the normal control group as the baseline; SC = sham-operated group; OC = ovariectomized (ovx) group; OE = ovx rats given estradiol benzoate (EB) 2.5 $\mu$g/kg BW/day; OJ10 = ovx rats given YCJ at 10 mL/kg BW/day; OJ20 = ovx rats given YCJ at 20 mL/kg BW/day; OJ40 = ovx rats given YCJ at 40 mL/kg BW/day.

### 3.2. Transmission Electron Microscopy (TEM): Keratinocytes

Figure 3 shows a transmission electron micrograph (TEM) of rat epidermis keratinocytes. According to the TEM analysis, the epidermal keratinocytes in the sham...
(SC), ovx+EB(OE), and ovx+YCJ (OJ) groups were tightly connected through desmosomes. The size of the keratinocyte cells in the OC group declined after ovariectomy (OC group). When the ovx rats were fed either EB (OE group) or YCJ (YJ group), cell and nucleus size increased (OJ groups). The desmosomes in the ovx epidermis were significantly less and were smaller than those in the control groups (SC and OE) and the OJ group. When compared to the OC group, the OJ group had more desmosomes and narrow intercellular gaps. The OJ group’s keratinocytes had the most cytoplasmic processes, and their numbers also increased. The above observations were confirmed when the statistical analysis was undertaken (Figure 4A,B). Surprisingly, when three doses of YCJ treatments were compared among each group, no significant differences in nuclei and keratinocyte cell perimeters were found (Figure 4A,B).

**Figure 3.** Electron micrograph (TEM) of the keratinocytes of rat skin epidermis (4000×). Note how the keratinocyte cells in the OC group have shrunk in size. The OJ groups had bigger nucleic and cytoplasmic processes than the OC group. More desmosomes and narrower intercellular spaces occurred in the OJ groups than in the OC group. Far fewer desmosomes and obvious intercellular spaces were observed in the OC group. NC = normal control (baseline) rats; SC = sham-operated rats; OC = ovariectomized (ovx) rats; OE = ovx rats given estradiol benzoate (EB) 2.5 µg/kg BW/day; OJ10 = ovx rats given YCJ at 10 mL/kg BW/day; OJ20 = ovx rats given YCJ at 20 mL/kg BW/day; OJ40 = ovx rats given YCJ at 40 mL/kg BW/day. The yellow arrows indicate desmosomes, and yellow chevrons indicate the intracellular spaces. Bar = 2 µm.
3.3. Transmission Electron Microscopy (TEM): Fibroblasts

The electron micrograph (TEM) of fibroblasts in the dermis was demonstrated in Figure 5. TEM examination demonstrated that the dermal fibroblasts of the normal control (SC, OE) groups were composed of large nuclei that contained more euchromatin than heterochromatin, and cytoplasmic processes were elongated without any dilation of the organelles, particularly the RER. Furthermore, there were numerous collagen fibers packed as large bundles surrounding the fibroblast cells. Following ovariectomy (OC group), the fibroblast cells in the OC group shrank in size; dilation of the rough endoplasmic reticulum (RER) in the cytoplasm was observed; cytoplasmic processes were smaller, and their numbers were also reduced. The reported dilation of the RER, as it appeared in the ovx group, is one of the indicators of cell degeneration. Cell size and nuclei size were increased when the ovx rats were treated with EB (OE group) or YCJ feeding (OJ groups). In the OJ groups, the nuclei were larger and the cytoplasmic processes were branching and elongated, compared with the OC group. In addition, the extracellular matrix had more numerous collagen fibers in the OJ groups, compared with the OC group. These observations were confirmed when statistical analysis of the collagen fibril numbers was performed (Figure 2D,E). The changes mentioned above were dose-related, with the OJ40 group representing the best dose for both in terms of the nucleus and the fibroblast cell perimeters (Figure 4C,D).
Figure 5. Electron micrograph (TEM) of rat skin fibroblast cells of the dermis (4000 ×). In the OC group, the fibroblast cell size was the smallest, and the dilation of RER (chevrons) in the cytoplasm was obviously present. The nuclei were larger in the OJ groups, and cytoplasmic processes were branching and elongating. In addition, in the OJ group, the extracellular matrix had more numerous collagen fibers than the OC group. NC = normal control (baseline) rats; SC = sham-operated rats; OC = ovariectomized (ovx) rats; OE = ovx rats given estradiol benzoate (EB) 2.5 µg/kg BW/day; OJ10 = ovx rats given YCJ at 10 mL/kg BW/day; OJ20 = ovx rats given YCJ at 20 mL/kg BW/day; OJ40 = ovx rats given YCJ at 40 mL/kg BW/day. The yellow arrows indicate collagen fibers, and yellow chevrons indicate the dilation of the rough endoplasmic reticulum (RER). Bar = 2 µm.

3.4. Transmission Electron Microscopy (TEM): Dermo-Epidermal Junction

The dermo-epidermal junction between the epidermis and dermis is shown in Figure 6. Basal borders at the dermo-epidermal junction of keratinocytes connected with the basement membrane via numerous hemidesmosomes. The basal borders of the cells were irregular, with protruding dermal papillary interdigitated with epidermal ridges. Following ovariectomy (the OC group), that junction was reduced compared to the control (NC, SC, and OE) and OJ groups. The collagen fibers in the papillary and reticular layers of the OC group were much smaller, shorter, and more loosely packed compared with the control (NC, SC, and OE) and OJ groups. In addition, the gap between the papillary and reticular layer of the OC group was wider, resulting in a wide space between those two layers. By contrast, the papillary layer and the reticular layers of the dermis for the control (NC, SC, and OE) and OJ groups contained numerous collagen fibers, packed tightly.
Figure 6. Electron micrograph (TEM) of the dermo-epidermal junction of rat skin epidermis (2000×). The junctions of rats treated with YCJ (OJ groups) were not different from the NC and SC groups. In contrast, the dermo-epidermal junctions of the OC and OE groups had markedly reduced irregularities of the basal borders compared to those of the NC, SC, and OJ groups. The papillary layer of the dermis had far fewer collagen fibers and was loosely packed. In the OC and OE groups, the spaces between papillary and reticular layers were wider compared with the NC, SC, and OJ groups. In addition, the collagen fibers of the reticular layer of the OC and OE groups were packed into much smaller bundles compared with the other groups. NC = normal control (baseline) rats; SC = sham-operated rats; OC = ovariectomized (ovx) rats; OE = ovx rats given estradiol benzoate (EB) 2.5 µg/kg BW/day; OJ10 = ovx rats given YCJ at 10 mL/kg BW/day; OJ20 = ovx rats given YCJ at 20 mL/kg BW/day; OJ40 = ovx rats given YCJ at 40 mL/kg BW/day. B = basement membrane, C = collagen fibers, D = dermis (yellow line), E = epidermis (white line), H = hemidesmosome. Bar = 2 µm.

3.5. Transmission Electron Microscopy (TEM): Collagen Fibrils

Figure 7 depicts the electron micrograph (TEM) of rat skin, showing an overview of the collagen fibers. Compared with the control (SC, OE) groups, the number of collagen bundles of the OC group was considerably smaller, and the spaces between bundles were wider following ovariectomy (OC group). The number of collagen bundles and their sizes were increased when the ovx rats were treated with EB (OE group) or YCJ feeding (OJ groups). Therefore, the space between the bundles of the OJ groups became narrower when compared with the SC, OE, and OC groups, as confirmed by the cross- and long sections.
(Figures 8 and 9, respectively). Those observations were confirmed when the collagen fibrils’ diameters in the thick sections of the dermis (Figure 2D) and the thin sections (Figure 2E) were measured. The changes mentioned above were dose-related, with the OJ40 group presenting the best results, even though there was no significant difference among all three YCJ treatments (Figure 2D,E).

**Figure 7.** Electron micrograph (TEM) of rat skin showing an overview of collagen fibers (2000×). The collagen fibers of the control (NC and SC) groups are arranged in parallel lines. Compared with the control groups, the collagen bundles of the OC group were much smaller. In contrast, collagen fibers combined in the YCJ (OJ) groups into large bundles, particularly that of the OJ40 group. Therefore, in the OJ groups, the space between the bundles (yellow arrows) became narrower when compared with the SC, OC, and OE groups. Yellow arrows indicate spaces between the collagen bundles. NC = normal control (baseline) rats; SC = sham-operated rats; OC = ovariectomized (ovx) rats; OE = ovx rats given estradiol benzoate (EB) 2.5 µg/kg BW/day; OJ10 = ovx rats given YCJ at 10 mL/kg BW/day; OJ20 = ovx rats given YCJ at 20 mL/kg BW/day; OJ40 = ovx rats given YCJ at 40 mL/kg BW/day. Bar = 5 µm.
Figure 8. Electron micrograph (TEM) of rat skin collagen fibrils of the dermis (cross-sections; 20,000×). The cross-sections show the ultrastructure of the collagen fibrils. The collagen diameters of the OC group were the smallest, and those of the OJ40 group were the largest. Furthermore, in the OC group, intercellular spaces between collagen fibrils were wider than in the controls (SC and OE groups) and in all three OJ groups (OJ10, OJ20, and OJ40 groups). NC = normal control (baseline) rats; SC = sham-operated rats; OC = ovariectomized (ovx) rats; OE = ovx rats given estradiol benzoate (EB) 2.5 µg/kg BW/day; OJ10 = ovx rats given YCJ at 10 mL/kg BW/day; OJ20 = ovx rats given YCJ at 20 mL/kg BW/day; OJ40 = ovx rats given YCJ at 40 mL/kg BW/day. Bar = 500 µm.
Figure 9. Electron micrograph (TEM) of rat skin collagen fibrils of the dermis (long sections; 20,000 ×). The characteristic alignment of the collagen fibrils is a parallel packing in all groups except that of the OJ group. Collagen fibril size in the OJ groups was larger than that of the OC group. In addition, the spaces between collagen fibrils of the OC group were wider compared with the other groups. NC = normal control (baseline) rats; SC = sham-operated rats; OC = ovariectomized (ovx) rats; OE = ovx rats given estradiol benzoate (EB) 2.5 µg/kg BW/day; OJ10 = ovx rats given YCJ at 10 mL/kg BW/day; OJ20 = ovx rats given YCJ at 20 mL/kg BW/day; OJ40 = ovx rats given YCJ at 40 mL/kg BW/day. Bar = 500 µm.

3.6. Ultrastructural Changes of Keratinocyte and Fibroblast Cell Scores

The ultrastructural changes of the keratinocyte and fibroblast cells were scored using the criteria shown in Table 1. When compared with the normal baseline (NC) group, after ten weeks of treatment, it was found that both the keratinocyte and fibroblast cell scores of the SC group were significantly reduced. The scores of the OC group were significantly lower, following ovariectomy. The scores were significantly increased when the ovx rats were treated with EB (OE group) or YCJ feeding (OJ groups), except in the case of the OE group of keratinocytes. For fibroblast cells, among three doses of YCJ treatment, the scores were not significantly different. In contrast with keratinocyte cells, ovx rats treated with YCJ 40 mL/kg BW (OJ40) presented the highest score compared to the three doses of YCJ treatment (Figure 10).
Table 1. Criteria for measurement of keratinocyte and fibroblast cell changes.

| Cells               | Complements                  | Degenerated Cell Features (0 Point)                  | Normal Cell Features (1 Point) |
|---------------------|------------------------------|-----------------------------------------------------|--------------------------------|
| Keratinocytes       | Intercellular space         | Widening of intercellular space                     | Non-widening of intercellular space |
|                     | Desmosome                   | Loss of desmosomes                                  | Many desmosomes                 |
|                     | Cell size                   | Reduced cytoplasmic area                            | Normal cytoplasmic area         |
| Dermoepidermal      |                              | Flat dermo-epidermal junction                       | The junction increased the number of footlike processes |
| junction            |                              |                                                     |                                |
| Fibroblasts         | Nucleus                     | -Irregular shape                                    | -Spindle shape                  |
|                     |                              | -Heterochromatin (Condensation of the nuclear chromatin) | -Increased euchromatin          |
|                     | Organelles                  | -Dilated of RER cisternae                           | -Normal RER                     |
|                     |                              | -Balooning of Golgi saccules                        | -Non-balooning of Golgi saccules |
|                     |                              | -Swollen mitochondria                               | -Non-swollen mitochondria       |
|                     | Cytoplasm                   | -Reduced cytoplasmic area                           | -Normal size of cytoplasm       |
|                     |                              | -Few branches                                       | -Branched and elongated         |
| Collagen fibrils    |                              | Few collagen fibrils around the fibroblasts          | Numerous collagen fibrils around the fibroblasts |

Figure 10. Ultrastructural changes of keratinocyte and fibroblast cell scores. Using the criteria as shown in Table 1, ultrastructural changes of keratinocyte and fibroblast cells were scored and statistically compared. All different superscripts indicate statistical significance at $p < 0.05$ level. NC = normal control (baseline) rats; SC = sham-operated rats; OC = ovarietomized (ovx) rats; OE = ovx rats given estradiol benzoate (EB) 2.5 $\mu$g/kg BW/day; OJ10 = ovx rats given YCJ at 10 mL/kg BW/day; OJ20 = ovx rats given YCJ at 20 mL/kg BW/day; OJ40 = ovx rats given YCJ at 40 mL/kg BW/day. Bar = 500 $\mu$m.
3.7. Gross Evaluation of Wound Healing

Among all six groups, the best accelerated wound healing and hair growth performance occurred in the OJ40 group. The hair was much softer and brighter in the OJ40 group, compared with the control and the OJ10 and OJ20 groups (Figure 11A,B). This beneficial effect of accelerating wound healing of YCJ confirmed our previous results and was persistent throughout every single experiment we conducted [18,19,23].

![Figure 11. (A) Gross morphology of wound healing for the sham-control group (SC), ovariectomized group (OC), and ovariectomized rats injected with estradiol benzoate(EB) 2.5 µg/kg BW/day group (OE), compared with that of YCJ (B) feeding groups (OJ): ovariectomized rats fed with YCJ 10 mL/kg BW/day (OJ10), ovariectomized rats fed with YCJ 20 mL/kg BW/day (OJ20), ovariectomized rats fed with YCJ 40 mL/kg BW/day (OJ40). The most accelerated wound healing and hair growth occurred in the OJ40 group. The number subscript indicates the following: 0 = 1 day after ovariectomy, 1 = 1 week after ovariectomy, 2 = 2 weeks after ovariectomy, 3 = 3 weeks after ovariectomy.](image)

4. Discussion

Skin aging and poor wound healing [24] are more common in menopausal women due to decreased estrogen production, which can impair the structure and function of the skin. HRT can help to counteract these side effects. However, the treatment may not be suitable for all menopausal women [13].

YCJ has been utilized in several medicinal applications but not for wound healing or for enhancing skin appearance. We were the first to report in 2006 that ovx rats given YCJ had significantly enhanced wound healing, including less scarring, brighter skin, and smoother hair, when compared to controls. However, these findings were based on a four-week period of gross morphological examination at the time. Later, we discovered that YCJ enhanced wound healing by downregulating the macrophage migration inhibitory factor (MIF) and via Erβ, rather than ERα [18]. To investigate these findings further, we conducted the current study, which examined changes in the skin, including its underlying structures, at a microscopic level (both LM and TEM) after rats were given YCJ over ten weeks, a longer time period than the original study. This was performed to simulate postmenopausal women’s prolonged consumption in an ovariectomized rat.

Even though the particular active components or phenolic groups of YCJ have yet to be determined, flavonoids make up most phytoestrogens with wound-healing activities [25]. As a result, we believe the positive effects of YCJ are attributable to its flavonoid properties. YCJ contains 58 percent beta-sitosterol, as well as additional sterols such as stigmasterol, stigmastatrienol, spinasterol, and fucosterol, as reported in previous studies [17]. According to Moghadasian et al. [26], beta-sitosterol may act as a sex steroid precursor since its structure is similar to animal cholesterol. Furthermore, the methanol extract of coconuts
.displayed an estrogenic effect in rats, due to its high concentration of beta-sitosterol, stigmasterol, and other flavonoids [27].

According to earlier studies [28–30], coconut extract is abundant in phytohormones like abscisic acid (ABA), auxin, gibberellins (GAs), and other cytokinins. In human fibroblast cells, trans-zeatin, one of the most significant cytokines contained in coconuts, has been demonstrated to have anti-aging characteristics [31]. According to this study, the rapid effect of YCJ on wound healing is mainly due to its strong estrogenic action, which increases the manufacture of endogenous estrogens.

Under LM and TEM examination, the current study discovered that YCJ increased skin thickness while preserving fibroblast and keratinocyte cell shape and increasing collagen fibril quantities. These findings are consistent with Uyar’s [32] study of the effects of soybean (Glycine max) extracts on the collagen layer and estrogen receptors in female rats’ skin. They discovered that soy isoflavones have positive effects on the skin through mechanisms such as lipid oxidation prevention, fibroblast proliferation stimulation, collagen degradation reduction, and the inhibition of 5-reductase [32].

Experiments with a cohort of postmenopausal women treated with isoflavone-rich soy extract for six months resulted in considerable increases in epithelial thickness, the number of elastic and collagen fibers, and the number of blood vessels [33]. The skin of ovariectomized rats administered red clover isoflavones appeared to be well-organized, with a normal epidermis of uniform thickness and regular keratinization and vascularity, collagen, and elastic fibers [34]. The amount of collagen in the red clover-isoflavones-treated group increased significantly compared to the control group [34].

The epidermis, dermis, and subcutaneous tissue comprise the three layers of skin. Keratinocytes make up most of the epidermis. Through estrogen receptors, phytoestrogens have an anti-aging impact on the skin [35]. In recent work, we discovered that YCJ consumption had a considerable effect on ERβ detection [18] and that Erβ stimulation leads to increased keratinocyte proliferation and migration [36]. Polito et al. found that treating ovx rats with genistein aglycone, a soy-derived isoflavone, enhanced collagen thickness via TGF-β1-producing cell types [37]. TGF-β1, a growth factor that promotes fibroblast proliferation and extracellular matrix (ECM) secretion, influences angiogenesis and epithelialization is the primary mechanism by which topical estrogen increases ECM secretion [38]. Our discovery of YCJ’s high binding to ERβ suggests that oxidative stress pathways may be involved in YCJ’s skin aging prevention activity in ovx rats. Many people believe that phytoestrogens can protect the skin from oxidative stress by binding to ERβ and activating ERE, to cause the dissociation of Nrf2 and Keap1. Nrf2 enters the nucleus and activates ARE, which increases the transcription of antioxidant enzymes [39]. This increases mitochondrial membrane potential and stimulates NO release in physiological circumstances. H2O2 induces a decrease in mitochondrial membrane potential, as well as an increase in NO production, which phytoestrogens can help to avoid. This helps to protect the skin from oxidative damage [40].

The outcome of hair follicle density in the dermis in this investigation reflects the findings of our prior study, in which we treated ovx rats with YCJ 100 mL/kg BW [18]. The current findings show that the number of hair follicles and their width in the dermis of the ovx+YCJ (OJ) group were higher and larger than the normal controls (NC, SC, OE) and the ovx (OC) groups utilizing lower dosages of YCJ of 10, 20, and 40 mL/kg BW. The number of hair follicles in the OJ groups grows in a dose-dependent manner, with the OJ40 (ovx+YCJ 40 mL/kg BW) group having the largest number and the ovx(OC) group having the lowest number. Lower dosages of YCJ (10, 20, and 30 mg/kg) were used in the current study. Hair follicles are important in skin biology for more than just their ability to produce hair. Since hair follicles are self-renewing and contain supplies of multipotent stem cells that may regenerate the epidermis, they are assumed to play a role in wound healing [41,42] and epidermal growth. Furthermore, positive correlations between the perimeters of the fibroblast, keratinocyte cells, collagen fibril diameters, and serum E2 levels reported in the current study confirmed the effects of YCJ on skin and skin appendages. This suggests that
some of the active ingredients in YCJ influence the growth and proliferation of fibroblasts, keratinocyte cells, and collagen fibrils.

The skin epidermis is a stratified squamous epithelium made up of four unique cell types, the most common of which are keratinocytes. The papillary layer is made up of loose connective tissue (fibroblasts, collagen fibers, and thin elastic fibers), and the reticular layer is made up of thick bundles of collagen fibers and coarse elastic fibers that create the skin dermis. Collagen is a fundamental part of the skin dermis, offering significant support for skin resistance, and is produced by fibroblasts. In the current study, statistical analysis revealed that estrogen and YCJ impacted both keratinocyte and fibroblast cells, resulting in increased collagen fibril formation and extracellular matrix content, as well as decreased intercellular space.

Collagen fibril diameters from both thick and thin sections of ovx rats receiving YCJ treatments in the current study were thicker than those of the control groups and the ovx group (Figure 8D,E), showing that both estrogen and YCJ are likely to influence collagen fibril diameters in ovx rats. Many studies have found that estrogen therapy improves collagen content and skin thickness [43–47]. Previous research involving a variety of phytoestrogens has backed up the current findings. For example, in human keratinocyte culture, Miyakazi et al. found that genistein and daidzein promote hyaluronic acid synthesis [48]. In a European trial of 234 postmenopausal women, isoﬂavone (Novadiol®) cream improved skin dryness and wrinkles after 12 weeks of treatment [49].

Raloxifene stimulates collagen production in human skin fibroblasts more efﬁciently than estradiol [50]. So far, phytoestrogens have been found to increase skin collagen thickness by: (1) inducing subcutaneous VEGF expression and increasing TGF-β in the skin [37,51]; (2) reducing collagen degradation by increasing TIMP protein levels to inhibit MMPs [52,53]; and (3) inhibiting FLT3 kinase activity and reducing Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase and Akt/p70 ribosomal kinase pathways, to inhibit AP-1 activity and reduce gene transcription of MMP-1 [54]. Additional molecular pathways need to be elucidated.

A single layer of columnar or high cuboidal keratinocytes rests on abasement membrane in the epidermis’ stratum basale. The basal domain of basal cells is anchored to the basement membrane by hemidesmosomes and associated intermediate ﬁlaments [42]. In the current study, the dermo-epidermal junctions of rats treated with YCJ (OJ groups) were not different from the NC and SC groups. The irregularities of the basal borders of the ovx(OC) and ovx+EB (OE) groups were markedly reduced compared with the control (NC, SC, and OJ) groups. This novel phenomenon indicates the obvious inﬂuences of YCJ on the protein component alteration of the hemidesmosomes of the stratum basale cell layer. Previously, it was found that phytoestrogens increase the production of hyaluronic acid [55] and extracellular matrix proteins [35], which gives rise to the hypothesis that it might help provide hemidesmosome structures in ovx rats treated with YCJ. However, the underlying molecular mechanisms need to be explored further in the future.

Both EB and YCJ considerably enhanced the thickness of the skin, both the epidermis and dermis, and the collagen content, as measured by collagen fibril diameters, and raised the number of hair follicle densities in ovx rats, according to the findings. These protective effects were validated histologically by LM and TEM levels, indicating that YCJ can prevent skin aging even in the absence of estrogen.

The skin is an estrogen-responsive tissue with specific receptors that respond to estrogen. While HRT has long been recommended to treat undesirable menopausal symptoms, as well as to prevent postmenopausal osteoporosis and other risk factors like heart attack and stroke, many studies have linked HRT to an increased risk of cardiovascular complications, venous thromboembolism, coronary artery disease [56], and many types of cancer [57–59]. As a result, the risks and benefits of systemic HRT have been thoroughly examined. Thus, HRT is not suggested for the treatment of skin aging, and topical estrogen cannot be used until clinical research has been completed to determine the minimum levels...
of estrogen compounds that have the best local effects without creating systemic hormonal side effects.

The question as to whether estrogen substitutes like phytoestrogens and SERMs are effective estrogens for mitigating skin aging in postmenopausal women has to be explored further. We examined a range of research on the effects of YCJ oral intake on the expression of ERα and ERβ in injured and normal skin [16,18,19], with the goal of determining if it has phytoestrogenic qualities and can operate as a SERM. Our findings show that YCJ, a non-steroidal plant with estrogen-like biological activity, appears to be a promising alternative to conventional hormone replacement therapy for skin aging without affecting liver or kidney function (please see Payanglee et al. for details on lipid, liver, and renal parameters [60]).

In summary, we have already shown that YCJ has a definite positive effect on cutaneous wound healing and skin appearance. These could be modified to have both therapeutic and cosmetic benefits, such as accelerating wound healing and reducing scars [18] from surgery, especially those associated with plastic surgery where scars must be greatly reduced, treating chronic ulcers, skin whitening, wrinkle reduction, preventing or reducing freckles, skin and hair diseases caused by estrogen deficiency, and repairing burns. The molecular processes involved, as well as the mechanisms through which YCJ and related substances regulate skin function and slow skin aging, are unknown at this time. More research will be required in the future to elucidate the issue.

5. Conclusions

The results of the current study indicated that: (1) EB injections at 2.5 µg/kg BW/day for 3 days per week was enough to restore aging skin back to its normal condition; (2) the effects of YCJ were similar to those of EB treatment in all parameters, with some parameters where YCJ was even better than EB; (3) at the various doses tested, YCJ treatment restored aging skin back to its normal condition with the optimal dose of 40 mL/kg BW/day (OJ40 group) with specific parameters, e.g., hair follicle numbers higher than all the normal control (NC, SC, and OE) groups; (4) the most accelerated wound healing and hair growth for YCJ occurred in the OJ40 group. These findings indicate that feeding YCJ could account, at least in part, for the protective role of estrogen replacement in preventing or reducing skin aging/damage in female rats: by increasing epidermal and dermal thickness; improving ultrastructural changes for keratinocytes and fibroblasts; increasing the number and size of collagen fibrils, and hence reducing wrinkles in ovx rats and enhancing skin complexion.

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Informed Consent Statement: This study did not involve with humans.

Data Availability Statement: The data used to support the findings of this study are included within the article.

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