

Original Article

A Comparative Study of the Effects of Raloxifene and Soy Extract on Skin Changes Induced by Ovariectomy in Rats

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A B S T R A C T

Background: Menopause due to estrogen deficiency accelerates age-related skin changes.

Aim of the Work: The present study was designed to compare the effects of raloxifene (a well known synthetic selective estrogen receptor modulator) with the natural phytoestrogen, soy extract, on skin changes in ovariectomized (OVX) rat model of menopause.

Methods: The animals were divided into sham group, OVX group, OVX treated with raloxifene (3 mg/kg/day) and OVX treated with soy extract (50mg/kg/day). The treatments were given via oral gavages for 4 weeks.

Results: Treatment of OVX rats with raloxifene or soy extract increased significantly collagen I gene and tissue inhibitor matrix metalloproteinase (TIMP)-1 gene in the skin with significant reduction in skin matrix metalloproteinase (MMP)-1 gene as compared to untreated OVX group. Raloxifene or soy extract treated OVX rats showed significant reduction in malondialdehyde (MDA) level in plasma with significant increase in plasma superoxide dismutase (SOD) enzyme activity and transforming growth factor-beta (TGF- β) level compared to the untreated OVX group. Histological investigations revealed that both drugs preserve epidermal thickness and restore collagen architecture in treated OVX rats compared to the untreated OVX group.

Conclusion: Soy extract was as effective as raloxifene in OVX rat model of menopause. Both treatments produced partial protective effects on the skin and these effects might be due to an estrogen like mechanism and the antioxidant properties.

Key Words: Raloxifene; Soy Extract; Skin; Ovariectomy; Albino Rats; Female

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1. INTRODUCTION

Menopause is often accompanied by atrophic changes of the skin resulting in thinner skin, an increase in number and depth of wrinkles, decreased collagen, increased skin dryness, decreased skin firmness and elasticity (Brincat, 2000).

Skin functions as an extracellular space that is predominantly comprised of fibrillar collagens, basement membrane, and elastin fibers constituting the extracellular matrix (ECM) and which gives skin its structural integrity (Philips et al., 2003). Atrophy of collagen and elastin fibers is primarily responsible for the morphological, functional and pathological diversity of skin tissue and result from the increased expression of their degradative enzymes. Collagen fibers are degraded by matrix metalloproteinase

(MMP) -1 and MMP-2 and the elastin fibers by MMP-2, MMP-9 and elastases. MMPs are a group of zinc-dependent extracellular proteinases which remodel the ECM and are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs), especially TIMP-1 and TIMP-2 (Philips et al., 2009).

Transforming growth factor- beta (TGF- β) is a multifunctional cytokine known to stimulate fibroblast proliferation in the dermis and induces the synthesis and secretion of the major ECM protein (Piek et al., 1999). In addition, TGF- β down regulates the expressions of proteolytic enzymes which degrade ECM proteins (Hall et al., 2003).

Estrogen therapy is used for attenuation of postmenopausal problems. Meanwhile, long term estrogen therapy increases the risk of uterine cancer and neoplasms of the breast (De-Lignières and MacGregor, 2000). Based on these criteria, increased attention has been placed on finding viable and safe alternatives. Selective estrogen receptor modulators (SERMs) are well established as synthetic estrogen substitutes. They were developed in an attempt to achieve the beneficial effects of estrogen, while minimizing the detrimental side effects in target tissues through specific estrogen receptor (ER) interactions (Verdier-Sévrain, 2007). These drugs exert mixed estrogenic and antiestrogenic effects depending on the tissue and cell type.

Raloxifene, one of the most studied synthetic SERMs, exhibits antiestrogen activity in the breast, lacks uterotrophic activity while having potentially beneficial estrogen-like effects in nonreproductive tissue, such as bone (Stygar et al., 2003). Phytoestrogens are natural nonsteroidal plant derived compounds. They structurally resemble endogenous estrogens so that they can directly bind to ERs and have tissue specific estrogen-like actions without the undesirable side effects (Accorsi-Neto et al., 2009). The major classes of phytoestrogens, of current interest, are isoflavones. Soy foods and supplements are rich sources for isoflavones. The primary soy-derived isoflavones are genistein, daidzein and glycitein (Kuiper et al., 1998).

The aim of the present work is to compare the effects of a naturally occurring SERM, soy extract, with raloxifene (a well known synthetic SERM) on skin changes in ovariectomized (OVX) rat model of menopause.

2. MATERIALS AND METHODS

2.1 Animals

A total of 40 adult healthy female Wistar rats weighing about 200-250g were used in this study. Animals were maintained under controlled environmental conditions (12 h light-dark cycle, temperature approximately 24°C, constant humidity 60±5%), and provided with standard food and water ad libitum. All animal procedures were performed after approval from the ethics committee of the National Research Centre Cairo, Egypt and in accordance with the international regulations for the use and care of experimental animals (Canadian Council on Animal Care Guidelines, 1993).

2.2 Drugs

Raloxifene hydrochloride (Lilly, Madrid, Spain) was supplied as white powder, dissolved in saline to be

given in a single dose of 3 mg/kg/day (Esposito et al., 2005). Soy extract (Mepaco, Egypt), one of the most commonly used dietary supplements, was supplied as powder, dissolved in distilled water to be given in a single dose of 50mg/kg/day (Gallo et al., 2005). Both treatments were administered orally via gastric tube for 4 weeks.

2.3 Surgical procedures

The rats undergo an aseptic surgical procedure as previously described by Ho et al. (2007). The rats were anesthetized using ketamine (100 mg/kg, IM) then the dorsal part of the lumbar region was shaved and the site cleaned with 75% ethanol. A 2 cm incision was made in the skin through the musculature and peritoneum and the ovaries were retracted and removed. The wound was then closed using a sterile suture and cleaned again with 75% ethanol to reduce the chance of postoperative infection. The sham operated group underwent the same surgical procedure except for the removal of the ovaries.

2.4 Experimental design

Rats were divided into four groups; each consisted of 10 rats as follows:

Group I (Sham operated control rats): Female rats underwent the above surgical procedures without the removal of the ovaries.

Group II (OVX rats): Female rats were subjected to bilateral ovariectomy as described previously.

Group III (OVX rats+ Raloxifene): Female rats were subjected to bilateral ovariectomy and on the next day they received oral raloxifene treatment (3 mg/kg/day) for 4 weeks.

Group IV (OVX rats+ Soy extract): Female rats were subjected to bilateral ovariectomy. After 24 hours, they received oral soy extract treatment (50 mg/kg/day) for 4 weeks.

At the end of the experiment (4 weeks), blood samples were taken from rat tail vein in collecting heparinized capillary tubes. The samples were centrifuged at 1000 Xg and plasma was split and frozen in polypropylene tubes at -20 °C until assayed. Animals from all groups were then sacrificed by decapitation, hair on the back was shaved, skin biopsy was performed and then part of the skin was blotted with a piece of filter paper, kept in foil paper and was kept at -70°C till used for further analysis of gene expression.

2.5 Biochemical analysis

2.5.1 Detection of collagen type I, MMP-1 and TIMP-1 gene expression by reverse transcription-polymerase chain reaction (RT-PCR):

RNA extraction:

Total RNA was extracted from skin tissue by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA content and purity was measured by using a Shimadzu UV spectrophotometer (Tokyo, Japan).

RT-PCR experiments:

RT-PCR was done using the extracted RNA for detection of collagen type I, MMP-1&TIMP-1 genes. For amplification of the targets genes, reverse transcription and PCR were run in two separate steps. Briefly, Reaction mixture of RT reaction containing 1 µg total RNA, 0.5 µg random primer, 5×RT buffer, 2.5 mmol/L dNTP, 20 U RNase inhibitor and 200 U MMLV reverse transcriptase in a total volume of 25 µl was incubated at 37°C for 60 minutes, then heated to 95 °C for 5 minutes to inactivate MMLV. PCR was carried out with 1.5 µl RT products, 10 × PCR buffer (without Mg²⁺) 2.5 µl, 2.0 µl dNTP (2.5 mmol/L), 2.0 µl MgCl₂ (25 mmol/L), 0.5 µl each primer (20 µmol/L) of β-actin, 0.5 µl each primer of gene to be tested (20 µmol/L) and 1 U of Taq DNA polymerase (Promega Corporation, USA), in a final volume of 25 µl. Thermal cycler conditions were as follows: a first denaturing cycle at 97 °C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96 °C for 1.5 min, annealing for 1.5 min, and extension at 72 °C for 3 min. A final extension cycle of 72 °C for 15 min was included. The appropriate primer pairs, annealing temperature were summarized in table, 1.

Agarose gel electrophoresis:

All PCR products were subjected to electrophoresis on 2% agarose stained with ethidium bromide and visualized by UV transilluminator (Figures 1, 2, 3, 4).

Semi-quantitative determination of PCR products:

Semi-quantitation was performed using the gel documentation system (BioDO, Analyser) supplied by Biometra (GmbH, Goettingen, Germany). According to the following amplification procedure, relative expression of each studied gene (R) was calculated following the formula:

$$R = \frac{\text{Densitometrical Units of each studied gene}}{\text{Densitometrical Units of } \beta\text{-actin}}$$

β-actin gene was used as an internal control such that data were standardized according to β-actin values.

2.5.2 Measurement of TGF-β

TGF-β was measured in plasma by using ELISA (Quantikine, R&D system, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5.3 Measurement of malondialdehyde (MDA)

The level of MDA, the product of lipid peroxidation, in the plasma was measured by the following procedure according to Yoshioka et al. (1979). 0.5 ml plasma was shaken with 2.5 ml of 20% trichloroacetic acid (TCA) in a 10 ml centrifuge tube. 1 ml of 0.6 % thiobarbituric acid (TBA) was added to the mixture, shaken, and warmed for 30 min in a boiling water bath followed by rapid cooling. Then it was shaken into a 4 ml of n-butyl-alcohol layer in a separation tube and MDA content in the plasma was determined from the absorbance at 535 and 520 nm by Shimadzu UV spectrophotometer (Tokyo, Japan) against butanol. The results were expressed as nmol/ml plasma.

2.5.4 Measurement of superoxide dismutase (SOD) enzyme

The activity of SOD enzyme in plasma was measured using SOD assay kit provided by Oxis research, USA. The Bioxytech SOD-525™ method is based on the SOD-mediated increase in the rate of autoxidation of 5, 6, 6a, 11b-tetrahydro-3, 9, 10-trihydroxybenzo-fluorene R1 in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. Interference due to mercaptans (RSH) such as reduced glutathione is controlled by pretreating samples with 1-methyl-2-vinylpyridinium R2, which directly eliminates mercaptans by means of a fast alkylation reaction. The kinetic measurement of the 525 nm absorbance change is performed after the addition of R1. The SOD activity is determined from the ratio of the autoxidation rates in the presence (Vs) and in the absence (Vc) of SOD. The Vs/Vc ratio as a function of SOD activity is independent of the type of SOD (Cu/Zn-SOD, Mn-SOD, Fe-SOD) being measured. One SOD-525 activity unit is defined as the activity that doubles the autoxidation rate of the control blank (Vs/Vc = 2) (Nebot et al., 1993).

2.6 Histological examination

Skin specimens were obtained from central dorsal region, flattened and fixed in 10% buffered formalin solution for 48 hours. The specimens were then dehydrated in ascending grades of ethanol and embedded in paraffin. Serial sections of 6 µm thickness were cut and stained with Haematoxylin-eosin (H&E) for routine histological examination (Kiernan, 2001), and Masson's trichrome to reveal skin collagen fibres (Jones et al., 2008).

2.7 Statistical analysis

All data are expressed as means \pm standard deviation (SD) for the quantitative variable. Analysis of variance (ANOVA) was performed on the means to determine whether there were significant ($P < 0.05$) differences among the groups. When ANOVA indicated statistical significance the Tukey-Kramer test follows up, for intergroup comparisons. GRAPHPAD Software (version 2.0, 1993, InStat, San Diego) was used for all statistical analyses. The results were considered significant when p value < 0.05 .

3. RESULTS

3.1 Biochemical results

3.1.1 Effects of Raloxifene and Soy extract on skin collagen type I gene expression

Compared with sham operated group (group I), ovariectomy (group II) induced a significant decrease ($p < 0.001$) in skin collagen I gene (1.5 ± 0.29 vs 0.3 ± 0.08 respectively, table, 2). Treatment of OVX rats with raloxifene (group III) induced a significant increase ($p < 0.001$) by 133% in skin collagen I gene as compared to untreated OVX group (0.7 ± 0.06 vs 0.3 ± 0.08 respectively, table, 2). Also soy extract treatment in OVX rats (group IV) induced a significant increase ($p < 0.001$) by 167% in skin collagen I gene as compared to untreated OVX group (0.8 ± 0.07 vs 0.3 ± 0.08 respectively, Table, 2). No statistically significant difference ($p > 0.05$) was detected in skin collagen I gene between group III and group IV at the end of the study but there was a significant difference ($p < 0.001$) in group III and group IV as compared to sham operated control (Table, 2).

3.1.2 Effects of Raloxifene and Soy-extract on skin MMP-1/TIMP-1 gene expression

In the present study, skin samples from untreated OVX rats (group II) is associated with significant increase ($p < 0.001$) in skin MMP-1 gene compared with sham operated group (0.8 ± 0.08 vs 0.3 ± 0.11 respectively, table, 2) together with significant decrease ($p < 0.001$) in skin TIMP-1 gene in OVX rats as compared with sham operated control rats (0.7 ± 0.24 vs 1.6 ± 0.35 , respectively, table, 2). OVX-rats treated with raloxifene (group III) showed significant decrease ($p < 0.001$) in skin MMP-1 gene by 37.5% compared with untreated OVX group (0.5 ± 0.09 vs 0.8 ± 0.08 respectively, Table, 2) together with significant increase ($p < 0.05$) in skin TIMP-1 gene by 71% as compared to untreated OVX group (1.2 ± 0.26 vs 0.7 ± 0.24 respectively, Table, 2). Also soy extract treatment in OVX rats (group IV) led to significant decrease ($p < 0.001$) in skin MMP-1 gene by 50% compared to untreated OVX group (0.4 ± 0.07 vs 0.8 ± 0.08 respectively) with significant increase ($p < 0.05$) in skin TIMP-1 gene by 86% as compared to

OVX group (1.3 ± 0.33 vs 0.7 ± 0.24 respectively, table, 2). There was no statistically significant differences ($p > 0.05$) in skin MMP-1 and TIMP1 genes between group III compared to group IV and between group IV (OVX +Soy extract) as compared to sham operated group (Table, 2). OVX-rats treated with raloxifene (group III) showed significant difference ($p < 0.05$) in skin MMP-1 gene expression with non significant difference ($p > 0.05$) in skin TIMP-1 gene expression compared to sham operated control (Table, 2).

3.1.3 Effects of Raloxifene and Soy extract on plasma TGF- β

In this study, estrogen loss following ovariectomy (group II) led to a significant decrease ($p < 0.001$) in plasma TGF- β level compared to sham operated control rats (27.1 ± 4.08 vs 60.2 ± 3.19 respectively, table, 2). The mean TGF- β level in plasma increased significantly ($p < 0.001$) in OVX rats treated with raloxifene (44 ± 1.87) and soy extract (43 ± 3.14) as compared to non treated OVX group (27.1 ± 4.08 , Table 2) This increase was by 62% and 59% respectively. There was no statistically significant difference ($p > 0.05$) in plasma TGF- β level between group III and group IV but there was significant difference ($p < 0.001$) between group III and group IV compared to sham operated control (Table 2).

3.1.4 Effects of Raloxifene and Soy extract on plasma oxidant/antioxidant system

In group II, ovariectomy induced a significant increase ($p < 0.001$) in plasma MDA level compared with sham group (19.7 ± 2.28 vs 11.2 ± 1.03 respectively, table, 2) together with significant decrease ($p < 0.001$) in plasma SOD activity in OVX rats as compared to control rats (0.2 ± 0.02 vs 0.7 ± 0.14 respectively, Table, 2). Treatment of OVX rats with raloxifene (group III) induced a significant decrease ($p < 0.001$) in plasma MDA level by 28% compared with non treated OVX rats (14.2 ± 1.41 vs 19.7 ± 2.28 respectively, Table 2) together with significant increase ($p < 0.01$) in plasma SOD activity by 100% in raloxifene treated as compared to untreated OVX rats (0.4 ± 0.08 vs 0.2 ± 0.02 respectively, Table, 2). Also, soy extract treatment in OVX rats (group IV) led to significant decrease ($p < 0.001$) in plasma MDA level by 26% compared with untreated OVX rats (14.6 ± 1.29 vs 19.7 ± 2.28 respectively, Table, 2) together with significant increase ($p < 0.01$) in plasma SOD activity by 100% in group IV as compared to untreated OVX rats (0.4 ± 0.11 vs 0.2 ± 0.02 respectively, Table, 2). There was no statistically significant difference ($p > 0.05$) in plasma MDA level and SOD enzyme activity between group III and group IV but there was significant difference ($p < 0.01$; $p < 0.001$ respectively) in these parameters between group III and group IV as compared to sham group (Table 2).

3.2 Histological results

H&E stained sections in this study, showed that the epidermal thickness was greatly reduced in untreated OVX rats (Fig. 5b) compared to sham operated control rats (Fig. 5a). Treatment of OVX rats with raloxifene in group III (Fig. 5c) or soy extract in group IV (Fig. 5d) preserve epidermal thickness as

compared to untreated OVX rats (Fig. 5b). The dermal collagen in the OVX group is scarce and disorganized (Fig. 6b) as compared to sham operated control group (Fig. 6a) while in group III treated with raloxifene (Fig. 6c) and group IV treated with soy extract (Fig. 6d), collagen bundles appear regularly arranged compared to untreated OVX group (Fig. 6b) as revealed by Masson's trichrome staining.

Table (1): Sequences of oligonucleotide primers

	Primer sequence	Annealing temperature
Collagen type I	Forward primer:5'- CAGGACCCTAACCCGGATGA -3' Reverse primer: 5'- CTCAAAGATGGTGTCTGCTGGT -3'.	65°C
MMP-1	Forward primer:5' CGGAGCACGGGGACGGGTATC -3' Reverse primer: 5' AAGACGAAGGGGAAGACGCACATC -3'.	55°C
TIMP-1	Forward primer:5'-TCTGGCATCCTCTTGTTGCT-3' Reverse primer: 5'-CACAGCCAGCACTATAGGTCTTT-3'	58°C
Beta actin	Forward primer:5'TGTTGTCCCTGTATGCCTCT-3' Reverse primer: 5'- 5'TAATGTCACGCACGATTTCC-3'.	60°C

Table (2): Effects of Raloxifene and Soy extract treatment on different biochemical parameters in OVX rats

Parameters	Control Group I	OVX Group II	OVX+Raloxifene Group III	OVX+Soy extract Group IV
Skin Collagen type- I gene	1.5±0.29	0.3±0.08*	0.7±0.06 ^{#*}	0.8±0.07 ^{#*}
Skin MMP-1 gene	0.3±0.11	0.8±0.08*	0.5±0.09 ^{#***}	0.4±0.07 [#]
Skin TIMP-1 gene	1.6±0.35	0.7±0.24*	1.2±0.26 ^{\$}	1.3±0.33 ^{\$}
Plasma TGF-β (pg/dl)	60.2±3.19	27.1±4.08*	44±1.87 ^{#*}	43±3.14 ^{#*}
Plasma MDA (nmol/ml)	11.2±1.03	19.7±2.28*	14.2±1.41 ^{#**}	14.6±1.29 ^{#**}
Plasma SOD (μ/ml)	0.7±0.14	0.2±0.02*	0.4±0.08 ^{##*}	0.4±0.11 ^{##*}

Values are expressed as means ± SD (n = 10 each group). **OVX:** ovariectomized, **MMP-1:** matrix metalloproteinase-1, **TIMP-1:** tissue inhibitor of matrix metalloproteinase-1, **TGF-β:** transforming growth factor-beta, **MDA:** malondialdehyde, **SOD:** superoxide dismutase. Analysis of variance (ANOVA) followed by Tukey-Kramer analysis was used for the comparison between the groups.

*p<0.001, ** p<0.01, *** p<0.05 vs control group

[#]p<0.001, ^{##}p<0.01, ^{\$}p<0.05 vs OVX group

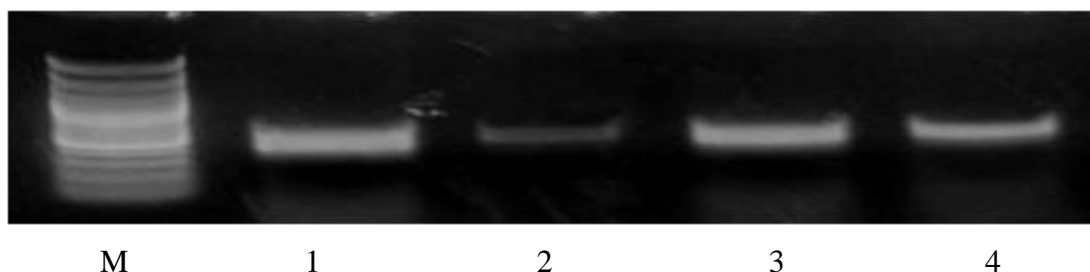


Fig. 1: Agarose gel electrophoresis shows PCR products of collagen type I gene expression in rat skin in the studied groups

Lane M: 100bp DNA ladder

Lane 1: PCR products of collagen type I gene in control group

Lane 2: PCR products of collagen type I gene in OVX group

Lane 3: PCR products of collagen type I gene in OVX+ Soy extract

Lane 4: PCR products of collagen type I gene in OVX+ Raloxifene

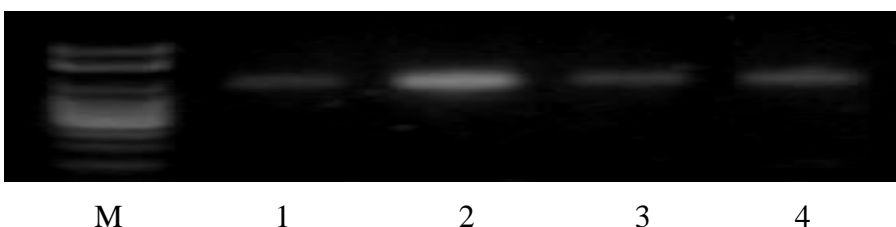


Fig. 2: Agarose gel electrophoresis showing PCR products of MMP-1 gene expression in rat skin in the studied groups

Lane M: 100bp DNA ladder

Lane 1: PCR products of MMP-1 gene in control group

Lane 2: PCR products of MMP-1 gene in OVX group

Lane 3: PCR products of MMP-1 gene in OVX+ Soy extract

Lane 4: PCR products of MMP-1 gene in OVX+ Raloxifene

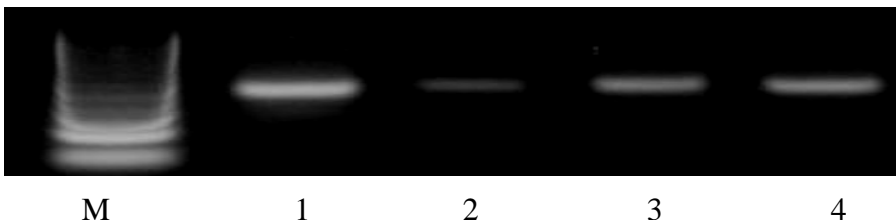


Fig. 3: Agarose gel electrophoresis showing PCR products of TIMP-1 gene expression in rat skin in the studied groups

Lane M: 100bp DNA ladder

Lane 1: PCR products of TIMP-1 gene in control group

Lane 2: PCR products of TIMP-1 gene in OVX group

Lane 3: PCR products of TIMP-1 gene in OVX + Soy extract

Lane 4: PCR products of TIMP-1 gene in OVX + Raloxifene

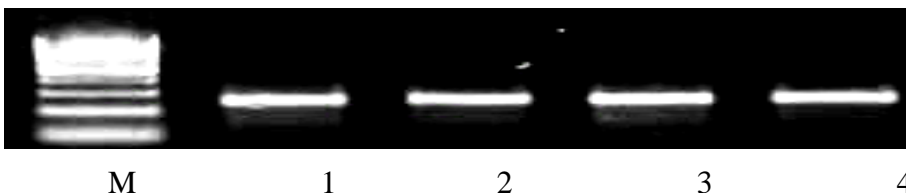


Fig. 4: Agarose gel electrophoresis showing PCR products of beta-actin gene expression (the control unit) in rat skin in the studied groups

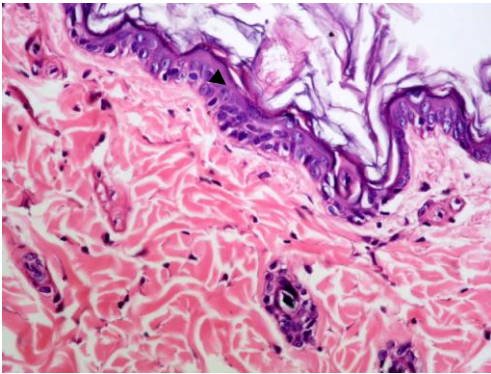
Lane M: 100bp DNA ladder

Lane 1: PCR products of beta actin gene in control group

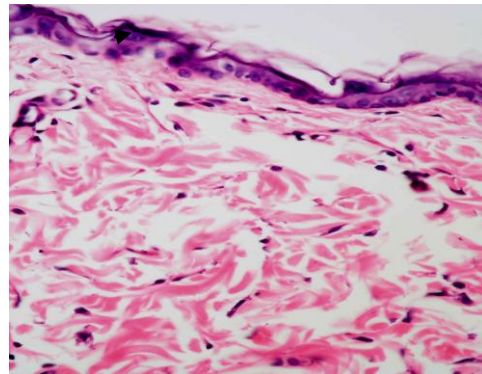
Lane 2: PCR products of beta actin gene in OVX group

Lane 3: PCR products of beta actin gene in OVX+ Soy extract

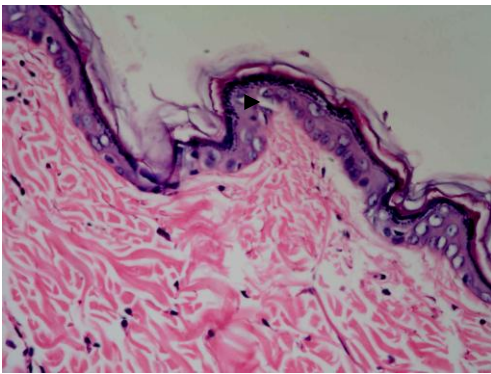
Lane 4: PCR products of beta actin gene in OVX+ Raloxifene



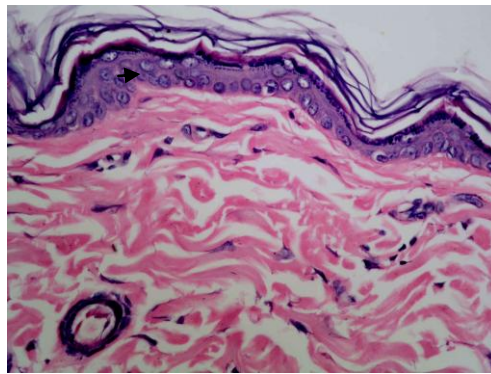
A



B



C



D

Fig. 5: Photomicrographs of the epidermis (**arrow heads**) from control group (A), OVX group (B), Raloxifene group (C) and Soy group (D). The epidermal thickness in the OVX group is greatly diminished in comparison to the other 3 groups (H&E X400).

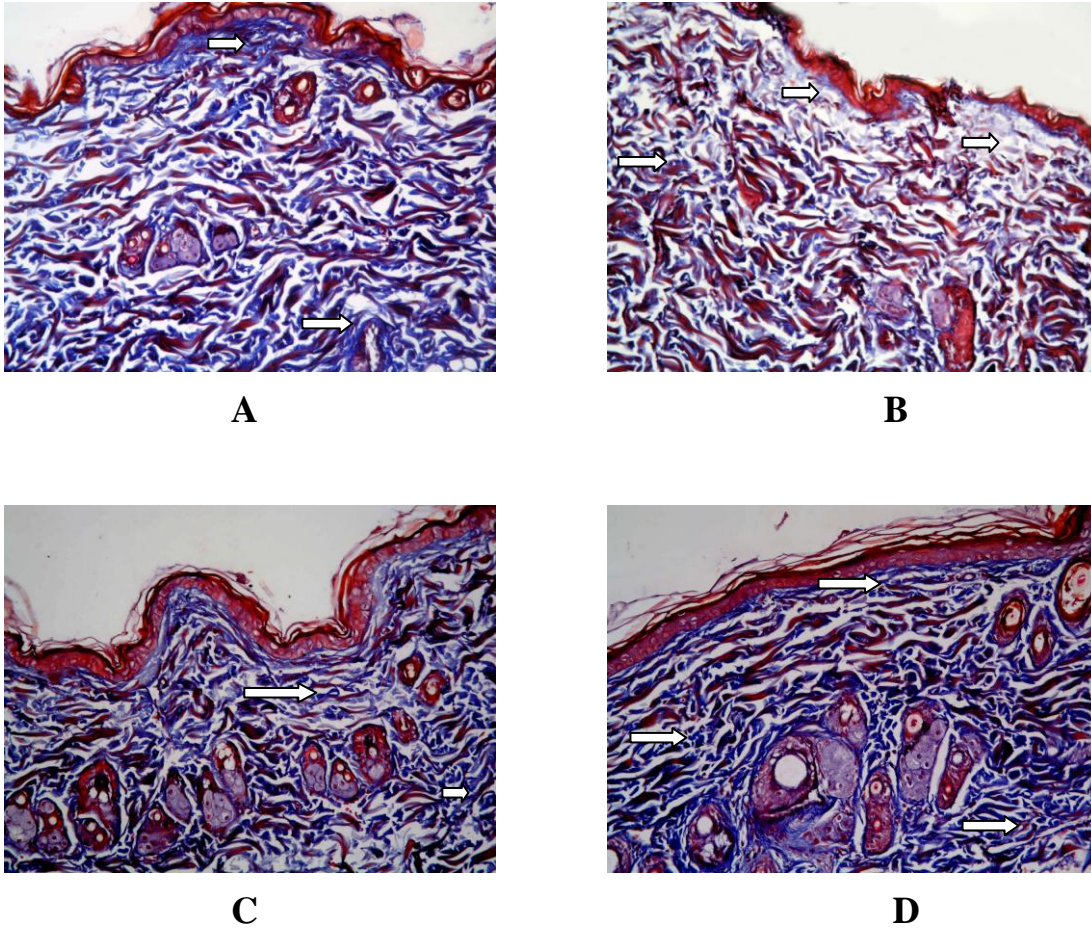


Fig. 6: Photomicrographs of skin tissue from control group (A), OVX group (B), Raloxifene group (C) and Soy group (D). The dermal collagen (arrows) in the OVX group is scarce and disorganized while in the other 3 groups, regularly arranged collagen bundles are clearly seen (Masson's trichrome X200).

4. DISCUSSION

Alterations in collagen, the major structural component of the skin, have been suggested to be a cause of the clinical changes observed in menopausal skin. The dermis contains predominantly type I collagen (85%–90%) with lesser amounts of type III collagen (10%–15%), synthesized by dermal fibroblasts and gives the dermis its mechanical and structural integrity (Uitto, 1986).

In the present study, estrogen loss following ovariectomy resulted in significant decrease in skin collagen type I gene expression in OVX rats compared to control rats. Consistent with our findings, hypoestrogenism decreased the collagen content of the skin in post-menopausal women (Brincat et al. 1983; Affinito et al. 1999) and in rat (Kafantari et al., 2000).

It is well known that adequate oestrogen levels are required to control structural integrity and functional capacity of the skin (Verdier- Sévrain et al., 2006). Skin tissue is an active target of estrogens, since ERs are expressed in epidermal keratinocytes and dermal fibroblasts where ER- α and ER- β co-express in the human skin dermal fibroblasts (Haczynski et al., 2002).

The amount of collagen in the dermis is controlled by type I procollagen de novo synthesis by dermal fibroblasts and its degradation by MMP-1 as stated by Varani et al. (2001). MMPs are a family of related zinc- containing proteinases that have the ability to degrade most ECM (Kähäri and Saarialho-Kere, 1999).

The mechanism by which estrogen increases ECM secretion was explained by Son et al. (2005) where they found that the topical application of 17 β -estradiol increased the expression of TGF- β , in aged human skin, and that neutralizing anti-TGF- β antibody can prevent increased type I procollagen production by 17 β -estradiol in cultured fibroblasts.

TGF- β is produced by dermal fibroblasts in response to estrogen and is a well known stimulator of collagen synthesis. TGF- β is known to stimulate fibroblast proliferation in the dermis and has been shown to upregulate the production of type I, III, and VII collagens and fibronectin (Massague, 1998). In addition, TGF- β down-regulates the expressions of proteolytic enzymes, including MMP-1, -2, -3, and -13 which degrade ECM proteins (Hall et al., 2003). In dermal fibroblast, TGF- β inhibits MMP-1 and stimulates collagen, MMP-2, and TIMPs (Philips et al., 2009).

Thus the significant decrease in plasma level of TGF- β observed in OVX rats in this study resulted in the decreased skin TIMP-1 gene expression and the increased skin MMP-1 gene expression and hence

excessive collagen degradation observed in OVX group.

Our data showed that plasma level of MDA, an end product of lipid peroxidation, is increased significantly together with significant decrease in plasma SOD enzyme activity in OVX rats compared to sham operated control rats. These results are consistent with the findings of Yalin et al. (2006). Estradiol is known to function as an antioxidant and as a free radical scavenger. In the absence of estrogen, oxidative stress is increased and some antioxidants are decreased and hence oxidative tissue damage increases (Muthusami et al., 2005).

In the current work our histological findings are in agreement with Circosta et al. (2006) who found that the thickness of the epidermis and the distribution and morphology of the collagen bundles were altered in OVX rats. Brincat (2000) stated that estrogen improves skin where the collagen content and quality is improved and skin thickness is increased.

SERMs (mixed estrogen agonists/antagonists) such as raloxifene and isoflavones are receiving attention for their potential role in skin health. In this study, raloxifene treatment for 4 weeks in OVX-rats preserves skin collagen as demonstrated by significant increase in skin collagen type I mRNA expression by 133% as compared to untreated OVX group. The previous results were in accordance and explained by Surazynski et al. (2003) where they found that raloxifene at 5 and 10 microM concentrations had greater stimulative effect on collagen biosynthesis in cultured human skin fibroblasts compared to estradiol. They stated that the increase of collagen synthesis induced by raloxifene may be activated by both ER dependent in which raloxifene binds with greater affinity to the ER- α , and independent pathways such as up-regulation of ERs, transcriptional regulation of collagen genes by ER-raloxifene complex or by inhibition of metalloproteinase expression.

In the current work, the significant increase in plasma TGF- β level by 62% in raloxifene treated OVX rats led to significant decrease in skin MMP-1 gene expression by 37.5% together with significant increase in skin TIMP-1 mRNA expression by 71% compared to untreated OVX rats. Consistent with the previous results, Polito et al. (2012) found that OVX rats treated with raloxifene showed increased TGF- β 1 and TIMP-1 in skin biopsies detected by western blot analysis compared to untreated OVX group.

In the present study, OVX rats treated with raloxifene showed significant decrease in plasma MDA level by 28% together with significant increase in plasma SOD enzyme activity by 100% compared to untreated OVX rats. These results suggest an antioxidant effect of raloxifene which are in

accordance with the results of Kaya et al. (2005) and Konyalioğlu et al. (2007).

Histological examination of OVX rat skin treated with raloxifene in the current work revealed that raloxifene preserved epidermal thickness and restored collagen architecture as compared to untreated OVX group. Similarly, Polito et al. (2012) found that raloxifene hydrochloride significantly increased collagen thickness and consequently, the architecture of skin was restored in OVX rats compared to untreated OVX animals.

In the current work, soy extract treatment for 4 weeks preserved collagen content in the skin in OVX rats compared to untreated OVX group by inhibiting collagen degradation. This was demonstrated by significant increase in skin collagen I gene expression by 167% with significant decrease in skin MMP-1 gene expression by 50% in soy extract treated group compared to untreated OVX group. Consistent with our findings, Kim et al. (2004) reported that the isoflavone treatment increased the amount of collagen deposition in the UV-damaged human fibroblast cultures and UV-damaged hairless mice model, which is partly due to the inhibitory effects on UV-induced MMP-1 expression and the subsequent collagen degradation.

The effects of soy extract observed in treated OVX rat skin in this study are due to the significant increase in plasma TGF- β by 59% compared to untreated OVX group. A study by, Marini et al. (2010) found that genistein aglycone administration showed a significant increase of TGF- β 1 expression detected by western blot analysis in treated OVX rats compared to untreated OVX group.

The results of the present study revealed that soy extract treatment in OVX rats resulted in significant decrease in plasma MDA level by 26% with significant increase in antioxidant SOD activity in plasma by 100% compared to untreated OVX rats. The previous findings suggest an antioxidant effect of soy extract which is in agreement with the results of Cai and Wei (1996). They have demonstrated that ingesting soy isoflavone (genistein) for 30 days significantly increases the activities of the antioxidant enzymes SOD, glutathione peroxidase, catalase and glutathione reductase in various mouse organs.

Isoflavones are hormone-like compounds from a dietary source which have beneficial health-related effects. They appear to exhibit biological activities similar to estrogen because they are heterocyclic phenol compounds with structural similarities to both natural and synthetic estrogens (Moutsatsou, 2007). The protective effects of isoflavones on the skin were explained by previous studies. Thornton et al. (2003) reported an estrogen-like mechanism where

isoflavones preferentially bind to and transactivate ER- β in comparison to ER- α and it is ER- β that is more widely distributed within the skin and skin structures. Also the findings of Huang et al. (2008) involve the participation of antioxidants for the protective effects of isoflavones on the skin.

The results presented here showed that OVX rats treated with soy extract increased epidermal thickness and preserved dermal collagen as assessed histologically compared to untreated OVX group. Supportive with our findings, Circosta et al. (2006) found that the skin of OVX rats treated with isoflavones appeared well organized with a normal epidermis with uniform thickness and well developed collagen fibers.

5. CONCLUSION

The results of the present study suggest that a natural phytoestrogen, soy extract, might be as effective as the synthetic SERM, raloxifene, on skin changes in OVX rat model of menopause. Both treatments appear to have **partial** protective effects on the skin in an experimental model of menopause which might be due to an estrogen like mechanism and the antioxidative property.

6. ACKNOWLEDGMENT

My deep thanks to Dr. Laila Ahmed Rashed, Professor of Medical Biochemistry, Faculty of Medicine, Cairo University for assisting me in the biochemical analysis. Special gratitude and thanks to Dr. Soheir Asaad, Professor of Histology, Faculty of Medicine, Cairo University for making the histological examination.

7. REFERENCES

- Accorsi-Neto, A., Haidar, M., Simões, R., Simões, M., Soares-Jr, J., Baracat, E., 2009.* Effects of isoflavones on the skin of postmenopausal women: A pilot study. *Clinics* 64 (6), 505–510.
- Affinito, P., Palomba, S., Sorrentino, C., Di Carlo, C., Bifulco, G., Arienzo, M.P., Nappi, C., 1999.* Effects of postmenopausal hypoestrogenism on skin collagen. *Maturitas* 33 (3), 239-47.
- Brincat, M.P., 2000.* Hormone replacement therapy and the skin. *Maturitas* 35(2), 107-117.
- Brincat, M., Moniz, C.F., Studd, J.W.W., Darby, A.J., Magos, A., Cooper, D., 1983.* Sex hormones and skin collagen content in postmenopausal women. *Br. Med. J* 287(6402), 1337-1338.
- Cai, Q., Wei, H., 1996.* Effect of dietary genistein on antioxidant enzyme activities in SENCAR mice. *Nutr. Cancer* 25(1), 1-7.

- Canadian Council on Animal Care (CCAC), 1993.** Guide to the Care and Use of Experimental Animals, Volume 1 (2nd Edition), Canada.
- Chomczynski, P., Sacchi, N., 1987.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem* 162(1), 156–159.
- Circosta, C., De Pasquale, R., Palumbo, D.R., Samperi, S., Occhiuto, F., 2006.** Effects of isoflavones from red clover (*Trifolium pratense*) on skin changes induced by ovariectomy in rats. *Phytother. Res* 20(12), 1096-1099.
- De Lignières, B., MacGregor, E.A., 2000.** Risks and benefits of hormone replacement therapy. *Cephalalgia* 20(3), 164-169.
- Esposito, E., Iacono, A., Raso, G.M., Pacilio, M., Coppola, A., Di Carlo, R., Meli, R., 2005.** Raloxifene, a selective estrogen receptor modulator, reduces carrageenan-induced acute inflammation in normal and ovariectomized rats. *Endocrinology* 146(8), 3301-3308.
- Gallo, D., Zannoni, G.F., Apollonio, P., Martinelli, E., Ferlini, C., Passetti, G., Riva, A., Morazzoni, P., Bombardelli, E., Scambia, G., 2005.** Characterization of the pharmacologic profile of a standardized soy extract in the ovariectomized rat model of menopause: Effects on bone, uterus, and lipid profile. *Menopause* 12(5), 589-600.
- Haczynski, J., Tarkowski, R., Jarzabek, K., Slomczynska, M., Wolczynski, S., Magoffin, D.A., Jakowicki, J.A., Jakimiuk, A.J., 2002.** Human cultured skin fibroblasts express estrogen receptor alpha and beta. *Int. J. Mol. Med* 10(2), 149-153.
- Hall, M.C., Young, D.A., Waters, J.G., Rowan, A.D., Chantry, A., Edwards, D.R., Clark, I.M., 2003.** The comparative role of activator protein 1 and Smad factors in the regulation of TIMP-1 and MMP-1 gene expression by transforming growth factor- β 1. *J. Biol. Chem* 278(12), 10304–10313.
- Ho, Y.J., Wang, C.F., Hsu, W.Y., Tseng, T., Hsu, C.C., Kao, M.D., Tsai, Y.F., 2007.** Psychoimmunological effects of dioscorea in ovariectomized rats: role of anxiety level. *Ann. Gen. Psychiatry* 6, 21.
- Huang, Z.R., Hung, C.F., Lin, Y.K., Fang, J.Y., 2008.** In vitro and in vivo evaluation of topical delivery and potential dermal use of soy isoflavones genistein and daidzein. *Int. J. Pharm* 364(1), 36-44.
- Jones, M.L., Bancroft, J.D., Gamble, M., 2008.** Connective tissues and stains. In: Bancroft, J.D., Gamble, M. (Eds.), *Theory and practice of histological techniques*. Church Livingstone Elsevier, Philadelphia (PA), pp. 135-160.
- Kafantari, H., Kounadi, E., Fatouros, M., Milonakis, M., Tzaphlidou, M., 2000.** Structural alterations in rat skin and bone collagen fibrils induced by ovariectomy. *Bone* 26(4), 349-353.
- Kähäri, V.M., Saarialho-Kere, U., 1999.** Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Ann. Med* 31(1), 34-45.
- Kaya, H., Ozkaya, O., Sezik, M., Arslanoglu, E., Yilmaztepe, A., Ulukaya, E., 2005.** Effects of raloxifene on serum malondialdehyde, erythrocyte superoxide dismutase, and erythrocyte glutathione peroxidase levels in healthy postmenopausal women. *Maturitas* 50(3), 182-188.
- Kiernan, J.A., 2001.** *Histological and histochemical methods: Theory and practice*. 3rd ed. London: Arnold, pp: 111-162.
- Kim, S.Y., Kim, S.J., Lee, J.Y., Kim, W.G., Park, W.S., Sim, Y.C., Lee, S.J., 2004.** Protective effects of dietary soy isoflavones against UV-induced skin-aging in hairless mouse model. *J. Am. Coll. Nutr* 23(2), 157-162.
- Konyalioglu, S., Durmaz, G., Yalcin, A., 2007.** The potential antioxidant effect of raloxifene treatment: A study on heart, liver and brain cortex of ovariectomized female rats. *Cell Biochem. Funct* 25(3), 259-266.
- Kuiper, G.G.J.M., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A. 1998.** Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* 139(10), 4252–4263.
- Marini, H., Polito, F., Altavilla, D., Irrera, N., Minutoli, L., Calò, M., Adamo, E.B., Vaccaro, M., Squadrito, F., Bitto, A., 2010.** Genistein aglycone improves skin repair in an incisional model of wound healing: A comparison with raloxifene and oestradiol in ovariectomized rats. *Br. J. Pharmacol* 160(5), 1185-1194.
- Massague, J., 1998.** TGF- β signal transduction. *Annu. Rev. Biochem* 67, 753–791.
- Moutsatsou, P., 2007.** The spectrum of phytoestrogens in nature: Our knowledge is expanding. *Hormones (Athens)* 6(3), 173–193.
- Muthusami, S., Ramachandran, I., Muthusamy, B., Vasudevan, G., Prabhu, V., Subramaniam, V., Jagadeesan, A., Narasimhan, S., 2005.** Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. *Clin. Chim. Acta* 360(1-2), 81-86.
- Nebot, C., Moutet, M., Huet, P., Xu, J.Z., Yadan, J.C., Chaudiere, J., 1993.** Spectrophotometric assay of superoxide dismutase activity based on the

activated autoxidation of a tetracyclic catechol. *Anal. Biochem* 214(2), 442-451.

Philips, N., Smith, J., Keller, T., Gonzalez, S., 2003. Predominant effects of *Polypodium leucotomos* on membrane integrity, lipid peroxidation, and expression of elastin and matrix metalloproteinase-1 in ultraviolet radiation exposed fibroblasts, and keratinocytes. *J. Dermatol. Sci* 32(1), 1-9.

Philips, N., Conte, J., Chen, Y.J., Natrajan, P., Taw, M., Keller, T., Givant, J., Tuason, M., Dulaj, L., Leonardi, D., Gonzalez, S., 2009. Beneficial regulation of matrix metalloproteinases and their inhibitors, fibrillar collagens and transforming growth factor- β by *Polypodium leucotomos*, directly or in dermal fibroblasts, ultraviolet radiated fibroblasts, and melanoma cells. *Arch. Dermatol. Res* 301(7), 487-495.

Piek, E., Heldin, C.H., Dijke, P.T., 1999. Specificity, diversity, and regulation in TGF- β superfamily signaling. *FASEB J* 13(15), 2105-2124.

Polito, F., Marini, H., Bitto, A., Irrera, N., Vaccaro, M., Adamo, E.B., Micali, A., Squadrito, F., Minutoli, L., Altavilla, D., 2012. Genistein aglycone, a soy-derived isoflavone, improves skin changes induced by ovariectomy in rats. *Br. J. Pharmacol* 165(4), 994-1005.

Son, E.D., Lee, J.Y., Lee, S., Kim, M.S., Lee, B.G., Chang, I.S., Chung, J.H., 2005. Topical application of 17 β -estradiol increases extracellular matrix protein synthesis by stimulating TGF- β signaling in aged human skin in vivo. *J. Invest. Dermatol* 124(6), 1149-1161.

Stygar, D., Muravitskaya, N., Eriksson, B., Eriksson, H., Sahlin, L., 2003. Effects of SERM (Selective Estrogen Receptor Modulator) treatment on growth and proliferation in the rat uterus. *Reprod. Biol. Endocrinol* 1:40.

Surazynski, A., Jarzabek, K., Haczynski, J., Laudanski, P., Palka, J., Wolczynski, S., 2003. Differential effects of estradiol and raloxifene on collagen biosynthesis in cultured human skin fibroblasts. *Int. J. Mol. Med* 12(5), 803-809.

Thornton, M.J., Taylor, A.H., Mulligan, K., Al-Azzawi, F., Lyon, C.C., O'Driscoll, J., Messenger, A.G., 2003. Oestrogen receptor beta is the predominant oestrogen receptor in human scalp skin. *Exp. Dermatol* 12(2), 181-190.

Uitto, J., 1986. Connective tissue biochemistry of the aging dermis: Age-related alterations in collagen and elastin. *Dermatol. Clin* 4(3), 433-446.

Varani, V., Spearman, D., Perone, P., Fligel, S.E., Datta, S.C., Wang, Z.Q., Shao, Y., Kang, S., Fisher, G.J., Voorhees, J.J., 2001. Inhibition of type I procollagen synthesis by damaged collagen in photoaged skin and by collagenase-degraded collagen in vitro. *Am. J. Pathol* 158(3), 931-942.

Verdier-Sévrain, S., 2007. Effect of estrogens on skin aging and the potential role of selective estrogen receptor modulators. *Climacteric* 10(4), 289-297.

Verdier-Sévrain, S., Bonté, F., Gilchrist, B., 2006. Biology of estrogens in skin: Implications for skin aging. *Exp. Dermatol* 15(2), 83-94.

Yalin, S., Comelekoglu, U., Bagis, S., Sahin, N.O., Ogenler, O., Hatungil, R., 2006. Acute effect of single-dose cadmium treatment on lipid peroxidation and antioxidant enzymes in ovariectomized rats. *Ecotoxicol. Environ. Saf* 65(1), 140-144.

Yoshioka, T., Kawada, K., Shimada, T., Mori, M., 1979. Lipid peroxidation in maternal and cord blood and protective mechanism against activated-oxygen toxicity in the blood. *Am. J. Obstet. Gynecol* 135, 372-376.