Chapter 6

Topical Equol: A Revolutionary Approach to Skin Anti-Aging (Validation via Both Gene and Protein Analysis)

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ABSTRACT

Cosmetic approaches targeting skin anti-aging to achieve a youthful appearance continue to emerge, especially with the advancements in technology and the exchange of scientific information where physicians and patients have a greater awareness of dermal biology. Traditional assays or protein analysis include cell and/or organotypic cultures to investigate the influence of a compound on human skin. Conversely, genetic strategies (gene array/mRNA levels) to identify and quantify gene expression are relatively new. Many companies only use gene expression science to make skin anti-aging claims which makes important assumptions without validation.

Our approach has been to utilize both gene analysis and protein analysis to examine various compounds for better validation of ingredients. For example, the mechanism of action for botanicals is now beginning to be revealed both at the protein and genetic levels in describing their ameliorating roles and functions in human skin. In this analysis these scientific approaches were employed to determine how effective the botanical equol is at improving skin anti-aging.

We believe that if we can understand how botanicals and other compounds positively influence gene expression (by stimulation or inhibition of certain genes) and compare this to protein expression, we can increase the gap between chronological and biological aging by enhancing extracelluar matrix (ECM) constituents of the dermis and epidermal components.

INTRODUCTION

There are literally thousands of products on the market designed to slow down skin aging. Unfortunately, many of these products do little more than moisturize the skin and plump fine lines on a surface level. As one of the latest advancements in aesthetic medicine, the polyphenolic/isoflavanoid equol goes beyond the skin's surface to re-direct skin aging on a structural and genetic level. Equol is a polyphenolic/isoflavanoid that has recently gained attention due to its potent antioxidant activity and high affinity for estrogen receptor β. It is these unique, simultaneous effects that make equol a revolutionary molecule for skin health preservation and a major contributing factor to dermal anti-aging.

Within the last 10-15 years, botanical compounds have been increasingly incorporated into cosmeceuticals, including protectant, whitening, anti-wrinkle, anti-aging and anti-oxidant agents.[1] One of the best known examples is resveratrol, a polyphenolic molecule with a wide range of positive uses in skin care and other products.[2] Equol, a polyphenolic/isoflavonoid compound found in white cabbage, several types of lettuces, and beans,[3] is now emerging as another exceptional botanical agent. Equol has powerful anti-oxidant properties superior to all of the other isoflavones and its unique molecular and biochemical messenger characteristics have implications for the treatment of age-related diseases.[3-5]

In addition to its potent free-radical quenching activity, equol's unique bio-regulatory effects on androgens further enhance its influence on healthy aging. Equol can bind to estrogen receptor β[6] in the epidermis and fibroblasts in the dermis and mimic the positive effects of natural estrogen. At the same time, it does not have a high affinity for estrogen receptor α, which has negative influences in skin as well as in prostate and breast tissue.[7] Moreover, equol can specifically bind the potent androgen, 5α-dihydrotestosterone (5α-DHT), but not other steroid molecules, and inhibit 5α-DHT's powerful negative actions in skin.[8-9] For instance, it is known that androgens decrease skin health by increasing the production of metalloproteinases (MMPs) that break down collagen and elastin and decrease wound healing; in contrast, estrogens are known to have positive influences on skin aging such as increasing collagen and elastin and decreasing MMPs.[10-13]

EQUOL AND SKIN ANTI-AGING

Equol Enhances Skin Health at the Genetic and Protein Level

To thoroughly understand the benefits of topical equol on skin aging, we took special care to evaluate its effects at the genetic and protein level.[9] Traditionally, cosmetic approaches to skin aging have been evaluated using cell and/or organotypic cultures (via protein analysis) to better understand the influence of a compound on human skin. Additionally, new genetic strategies which consider gene array/mRNA levels have been introduced. Though a majority of genes that are stimulated translate and then transcribe their encoded sequences into proteins, only relying on gene expression science may or may not reveal the true story of functional or detectable protein expression and hence skin anti-aging properties. Thus, it is important to consider the following questions: If a gene is stimulated and then transcribed into protein, is there good correlation between gene activation and protein expression or abundance? Is relying only on genetic expression science a valid approach to functional outcomes in skin anti-aging characteristics? In evaluating the benefits of topical equol on skin-aging, we decided to use both scientific approaches to determine if a botanical molecule displays this correlative expression at the gene/mRNA and protein levels for several skin genes/proteins that were followed by validation via topical application in humans (Figure 1).

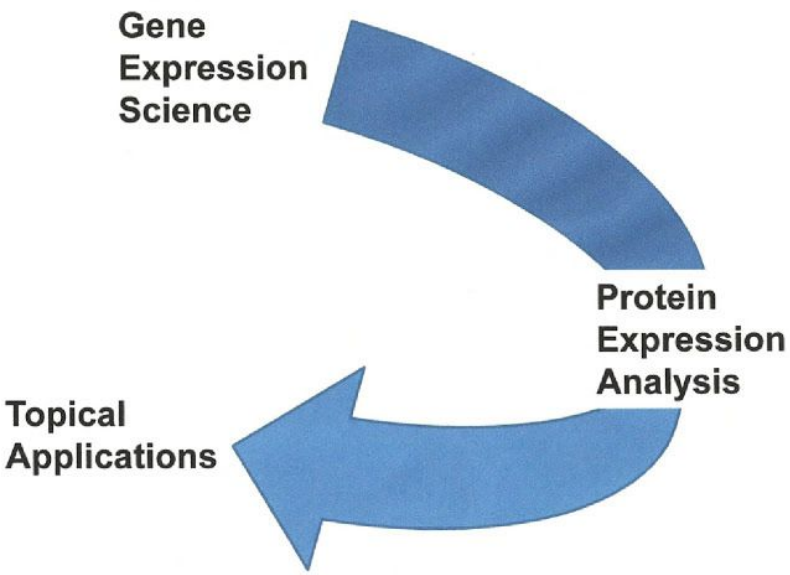


Figure 1. Approach strategy: starting with gene expression science to determine which genes are stimulated or inhibited provides a view of transcriptions regulation followed by gene product or protein expression analysis. If the genetic and protein expression results match up then steps to move forward with topical applications are validated.

Though we have performed gene analyses and biochemical (protein expression) investigations on resveratrol and many resveratrol analogs (and this strategy can be employed for any chemical or molecule), in this study we used equol as a prototype to better understand how it can enhance skin health. Thus, a comprehensive study was performed on equol to determine effective topical strategies that favor skin protection, regeneration, and rejuvenation. This was accomplished through a series of investigations using gene (array/mRNA level) expression where several skin genes could be examined at the same time using a human skin equivalent or dermal barrier.[9] We also utilized traditional cell culture techniques to quantify extracellular matrix (ECM) proteins like collagen and elastin that are known to be essential for good skin maintenance and health, especially with regards to aging.[9] Finally, using human cell cultures, we quantified ECM proteins that are detrimental to skin health such as the MMPs that break down collagen and elastin.[9]

Skin Penetration: Equol Forms a "Natural Reservoir" Delivery Mechanism for Optimal Release into the Skin

Gene expression analysis provides a direct view of transcriptional activity and regulation, which is a useful complement to analysis at the level of proteins. This technology enables the testing of several to hundreds of genes using epidermal full thickness (EFT) human skin cultures via gene array/ mRNA analysis. Since the EFT cultures represent a human skin barrier equivalent, compounds can be tested for their ability to penetrate the multiple-cell layers of the epidermal/dermal components and stimulate or inhibit gene expression (by the quantification of mRNA levels). In turn, the gene array data can be compared to in vitro cell culture results of human dermal monolayer fibroblasts in short-term (48-hour) or long-term (6- to 8-week) organotypic cultures that recapitulate the protein ECM dermal components found in human skin.[14,15] These technologies provide valuable data sets to reveal and compare gene versus protein expression that can lead to topical applications.

We knew from preliminary skin penetrating studies using human skin in the Franz cell model that equol was sequestered within and then released from the keratinocytes in the epidermis which contain a high level of estrogen receptor β.[16] This is an extraordinary situation where a skin agent such as equol is trapped within the keratinocytes and then is slowly released into the dermal layers. This provides "a natural reservoir" delivery mechanism to influence skin health in a positive manner over time and with each topical application (Figure 2).

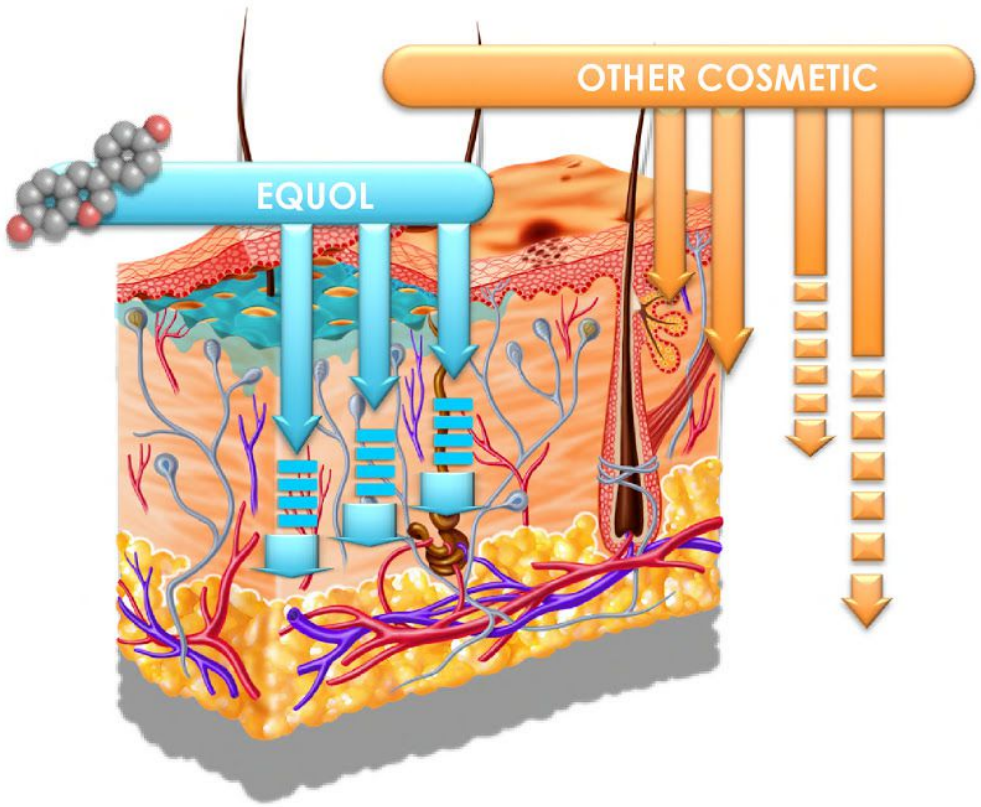


Figure 2. Franz cell testing demonstrates the "pooling" of equol in the epidermis (keratinocytes) forming a "natural reservoir." Unlike other cosmetics, equol is released from this "natural reservoir" into the dermis over time for optimal fibroblast function.

Equol Significantly Stimulates Collagen, Elastin, and TIMP 1, and Inhibits Skin Aging Gene Expression: Array/mRNA level Analysis

Since several dermal genes can be examined at one time using gene array technology, we used human skin equivalents to quantify gene expression when equol was applied to the cultures for 24-hours. When we tested 44 skin-related genes, more than half of the genes tested displayed positive results. Only a subset of the data is presented here. The results from this aspect of the study were

impressive. Equol significantly stimulated antioxidant genes (e.g., metallothionein-1H and metallothionein-2A, thioredoxin reductase 1, and superoxide dismutase 2) over control values that ranged from 200% to 1,800%. At the same time, S100 A8 and S100 A9, the genes that increase aging, were significantly decreased by 1000% and 1500%, respectively. When the skin ECM proteins were examined in the same human skin equivalent culture system, collagen type I was significantly increased by 220%, elastin was increased by 190%, and tissue inhibitor of metalloproteinase 1 (TIMP1) was increased by 540% (TIMP1 inhibits the actions of MMPs). Conversely, MMP 1, MMP 3 and MMP 9 were significantly inhibited by 540%, 800%, and 1100% respectively (Figure 3).

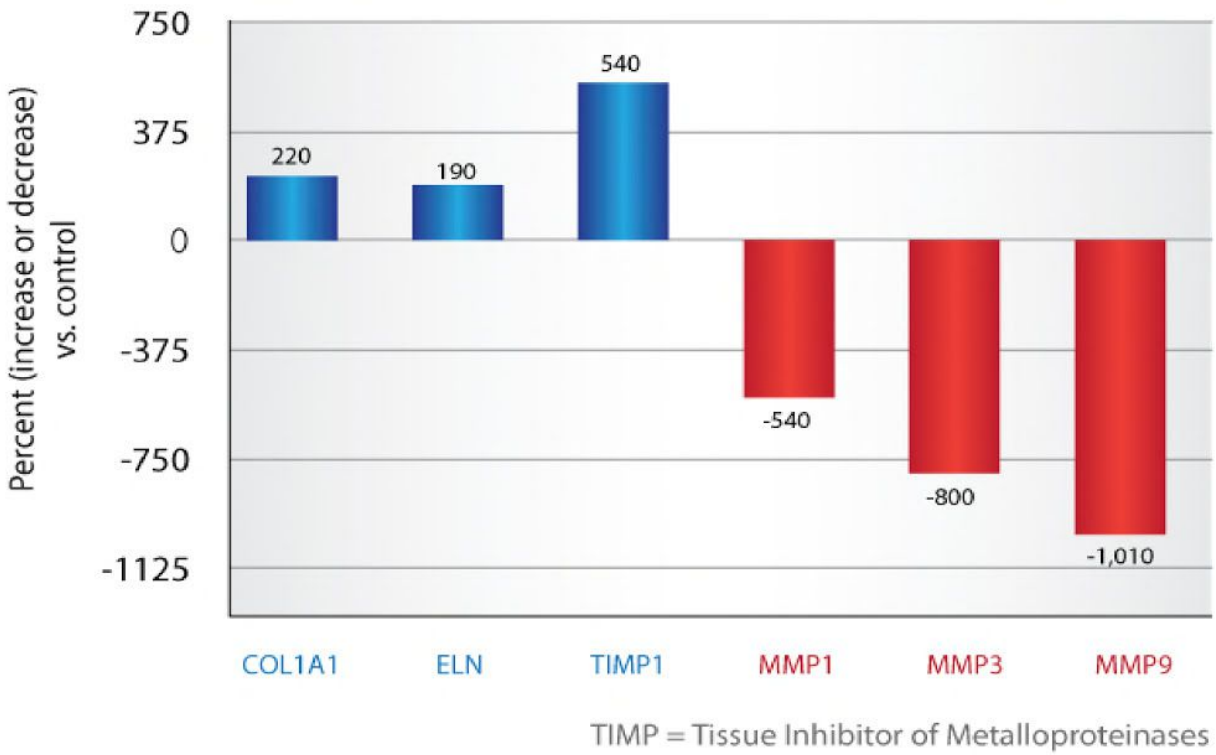


Figure 3. Equol significantly stimulates collagen, elastin, and TIMP 1, and inhibits MMP-1, MMP-3, and MMP-9 via gene array/mRNA analysis using human skin barrier equivalents (epidermal full-thickness cultures) versus control values (n = 6, expressed as the mean, 24-hour exposure).

The primary efficacy outcome of these data indicate that equol significantly stimulates genes such as collagen, elastin, and TIMP1 and antioxidant genes that provide good dermal integrity, improve functional turgor, and enhance skin health while at the same time significantly inhibiting detrimental ECM proteins like MMPs and S100. Finally, not only are the known powerful antioxidant properties of equol confirmed in this gene analysis, but this study also shows that equol is capable of modulating gene expression in human skin in dramatic and previously unknown positive ways to facilitate the anti-aging mechanisms in skin similar to that of natural estrogens.

Equol Increases Collagen While Blocking the Negative Impact of 5α-DHT

In short-term cell cultures (48-hours) using human monolayer dermal fibroblasts, equol, 5α-dihydrotestosterone (DHT), and 17β-estradiol at 10 nanomolar were studied for ECM protein expression. Equol significantly increased collagen type I by 1.8-fold (data not shown graphically), whereas 5α-DHT significantly decreased cell viability (Figure 4). When equol was applied along with 5α-DHT, cell viability was restored to normal levels, suggesting that equol blocked the negative impact of 5α-DHT, presumably by binding this potent androgen and biologically inactivating it.

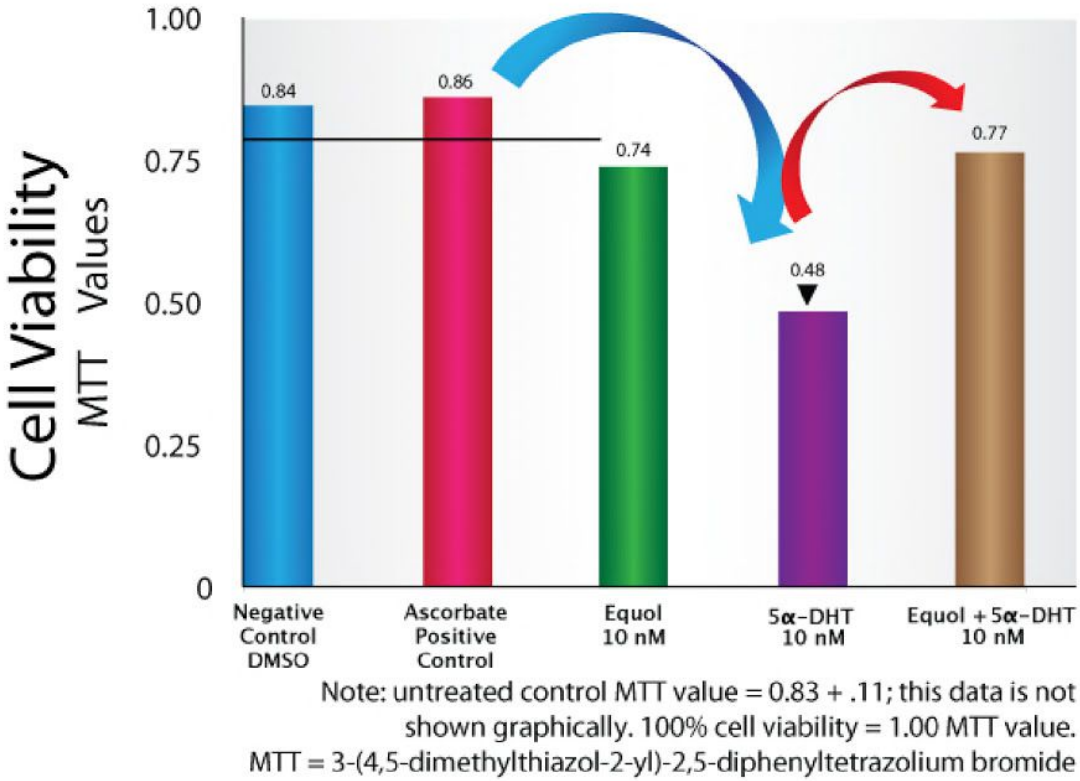


Figure 4. Equol reverses the toxicity of 5α-DHT in primary human monolayer fibroblast cultures back to control levels (n = 4, expressed by the mean, 48-hour exposure).

In long-term organotypic 3-dimensional cell cultures (6-8 weeks) using human dermal fibroblasts (that recapitulate the ECM dermal components found in human skin), intracellular fluorescent-activated cell sorting (FACS) analysis was employed where individual cells were identified and quantified by human antibodies to ECM proteins like collagen, elastin, elastase, and MMPs. Confirming the gene science results, in general, equol and 17β-estradiol significantly increased collagen type I, collagen type III, and elastin protein expression, while elastase (the enzyme that breaks down elastin) and MMP 1 and MMP 3 were significantly reduced (Figure 5A and 5B). These ECM proteins are essential for dermal structural integrity and the reduction in fine to medium lines and wrinkles to yield a youthful appearance of the skin.

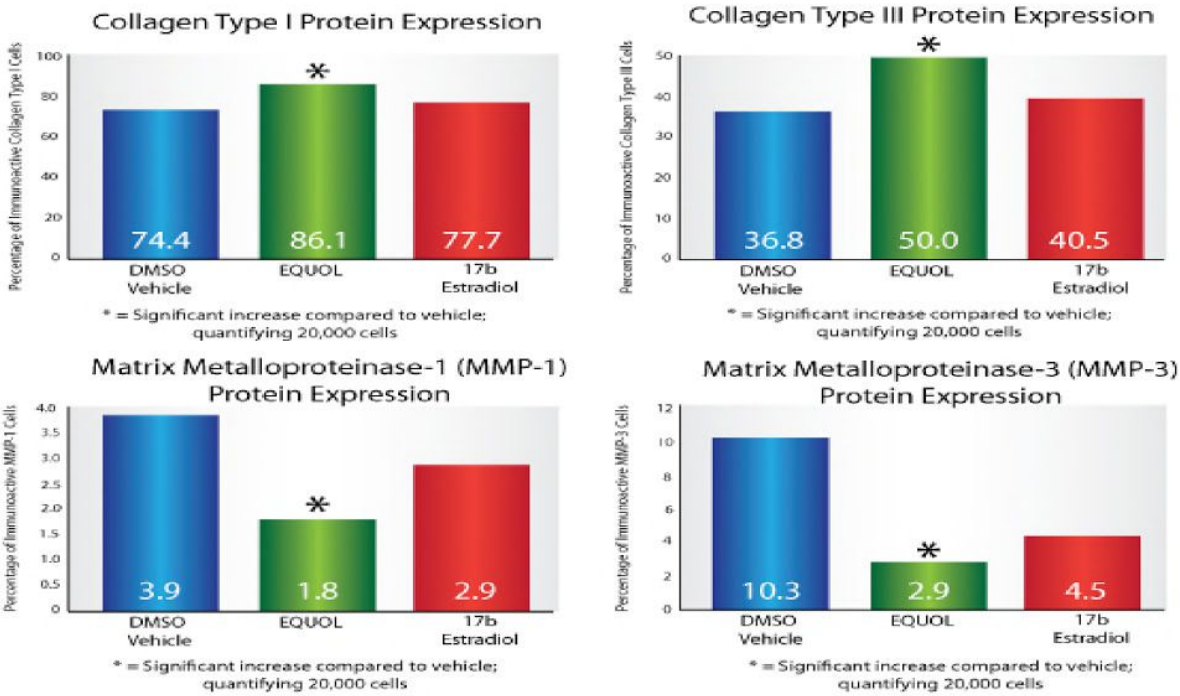


Figure 5A. Equol significantly stimulates collagen type I and type III and significantly inhibits MMP-1 and MMP-3 in long-term human skin cultures. All treatments = 10 nanomolar (nM) exposure for 4 days at the end of the cultures; n = 5 for each extracellular matrix (ECM) protein assayed, expressed as the mean.

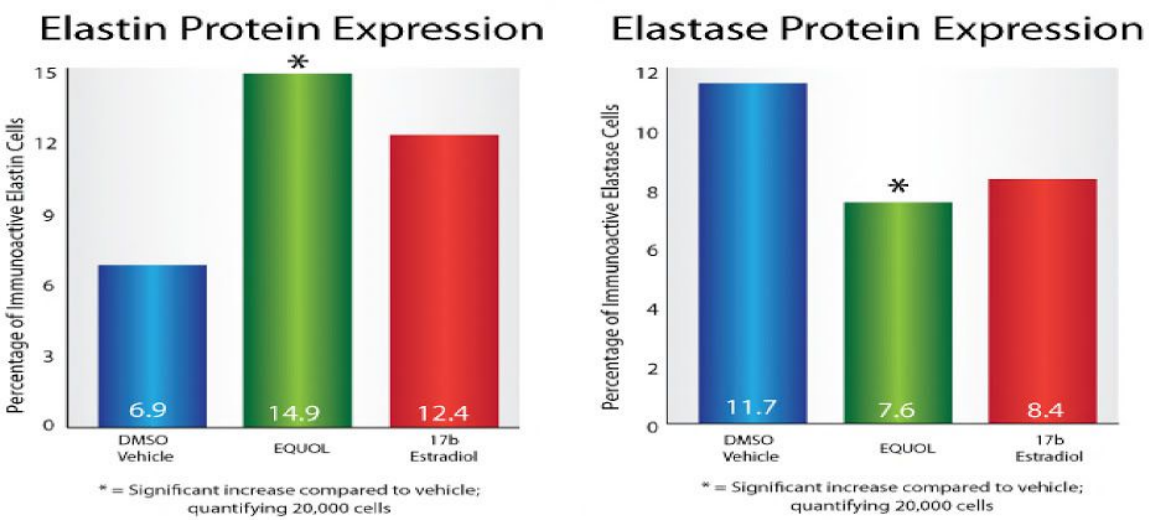
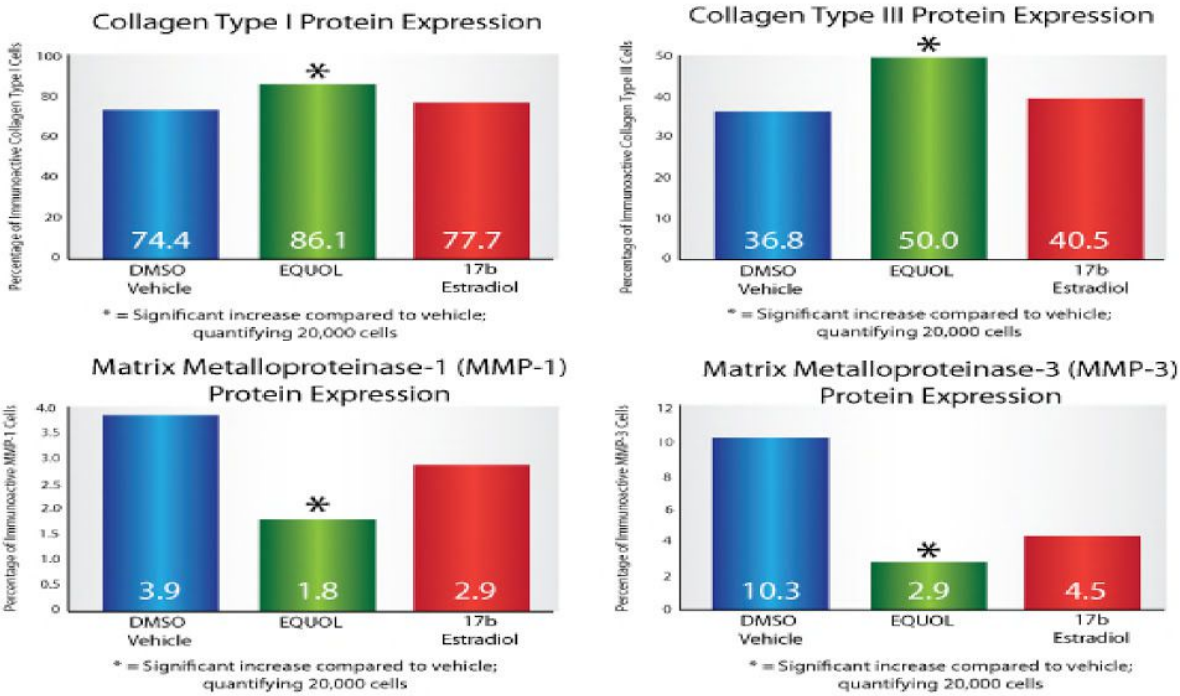


Figure 5B. Equol significantly stimulates elastin and significantly inhibits elastase in long-term human skin cultures. All treatments = 10 nanomolar (nM) exposure for 4 days at the end of the cultures; n = 5 for each extracellular matrix (ECM) protein assayed, expressed as the mean.

One goal of the long-term experiments was to determine how ECM protein expression is positively influenced by equol along with using the natural steroid hormone, 17β-estradiol, as a positive control. In this regard equol outperformed 17β-estradiol presumably due to its affinity for estrogen receptor β only, while 17β-estradiol has almost equal affinity for both estrogen receptors α and β. Another goal was to perform DNA analysis to determine whether equol or 17β-estradiol influenced cell cycle characteristics such as synthesis or arrest, etc. Remarkably, at 10 nanomolar, equol significantly increased fibroblast renewal by 1.5-fold compared to control or 17β-estradiol values. The final goal for the FACS analysis was to determine, in part, the mechanism by which equol exerts its positive actions. This was done by using the estrogen receptor blocker tamoxifen. Treatment with tamoxifen abolished



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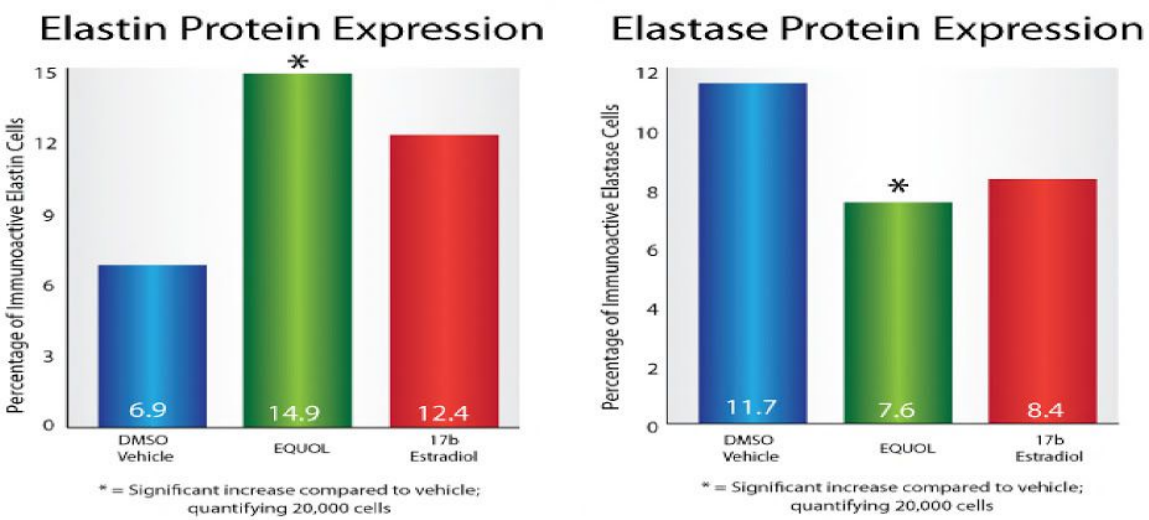

Figure 5B. Equol significantly stimulates elastin and significantly inhibits elastase in long-term human skin cultures. All treatments = 10 nanomolar (nM) exposure for 4 days at the end of the cultures; n = 5 for each extracellular matrix (ECM) protein assayed, expressed as the mean.

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the positive influences of equol on ECM protein expression (Figure 6). This suggests that equol not only has a beneficial influence on dermal cell viability and function to stimulate ECM proteins in many positive ways, but that equol also rejuvenates dermal fibroblasts for a healthier skin barrier and turgor. The mechanism by which equol imparts increased skin health appears to be via estrogen receptor β, at least in part, in epidermal and dermal areas of the skin.

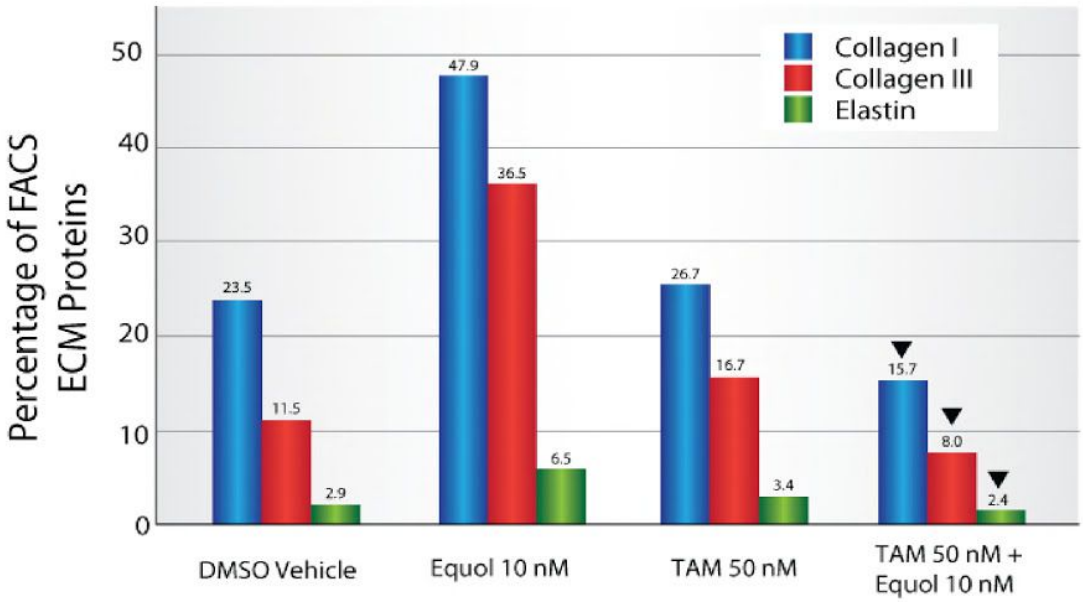

Figure 6. Tamoxifen inhibits equol's stimulation of collagen type I, collagen type III, and elastin protein expression in 6-week human skin cultures via fluorescent activated cell sorting (FACS) analysis, suggesting that equol acts through estrogen receptor β to yield its powerful skin enhancing effects.

However, recent data suggest that equol specifically can bind to and activate the orphan nuclear estrogen related receptor (ERR) gamma that is present in human keratinocytes and fibroblasts.[17,18] This orphan nuclear receptor has a similar structure to the nuclear estrogen receptors but does not respond to estrogens. The presence of ERR-gamma during prostate cancer is indicative of a favorable prognosis, and ERR-gamma has been shown to slow proliferation in prostate and breast cancer cell lines.[17,19] Using in vitro cultures examining PC-3 cells, equol has been shown to increase the transcriptional activity of ERR-gamma, thereby enhancing the inhibitory actions of ERR-gamma on neoplastic growth. The concentrations of equol required to stimulate ERR-gamma are high but not out of physiological range if equol were applied topically or consumed orally. Based on these findings, the beneficial effects of equol may be mediated not only through estrogen receptor β, but also estrogen-related receptor gamma in human skin.

In summary, taking into account all of the positive actions equol has in skin, it appears that the primary efficacy outcome of these data indicate that: Equol significantly stimulates ECM protein expression such as collagen subtypes and elastin, while at the same time significantly reducing ECM protein expression of elastase and MMPs. Also, it is important to point out that a comparison of the gene expression data matches up nicely with the protein expression results and provides a promising avenue to incorporate equol in topical applications for skin anti-aging.

Equol is Safe

Safety and toxicology testing has been conducted on equol as a single agent or in final formulas via preclinical ocular/dermal Irritection™ and EpiOcular™ model assays where it was found to be non-irritating. Additionally, human repeat insult-patch testing (RIPT) in several large panels of subjects yielded non-irritating or non-sensitization results. Finally, testing of equol via dermatology grading and safety/allergy assessments support the positive results shown by the gene and protein expression data.

Equol as an Anchor Active Ingredient in Cosmetics for Incorporation into Commercial and Clinical Products

A topical cosmetic containing equol as an active ingredient from a major cosmetic company was awarded "The Best Anti-Wrinkle Serum" by New Beauty magazine in its winter 2012 issue. Winners were chosen by thousands of avid skin care consumers and experts. However, this major cosmetic company does not distribute via regular retail/wholesale channels.

Equol is currently available only to practitioners as a key ingredient in yü InfiniSerum, which can be incorporated into clinical practice.

CONCLUDING REMARKS

Gene expression analysis provides a direct view of transcriptional activity and regulation. When gene expression science is combined with protein expression analysis using human skin equivalents and human skin cell cultures, these technologies provide valuable data sets to reveal and compare gene versus protein expression leading to topical applications. Though this science-based approach can be employed to examine any chemical or molecule, we used equol, an isoflavonoid botanical, as a prototype to better understand how this compound may enhance skin health. The obtained data suggest that equol has the potential to address the chronological, intrinsic, and extrinsic elements of skin aging by enhancing ECM components in the skin, based on these findings:

1) Equol stimulates collagen type I, collagen type III, and elastin protein production, while downregulating MMPs;
2) Equol increases the expression of skin antioxidants, relevant for protection against skin damage;
3) Equol has the ability to restore skin's components in a positive manner similar to 17β-estradiol by preventing the skin-damaging effects of androgens and binding to 5α-DHT;
4) Equol has affinity for estrogen receptor β indicating that it possesses similar benefits of natural estrogen by facilitating gene and protein expression and the biological mechanisms favorable for skin anti-aging.

A summary of how equol works in skin is shown in Figure 7.

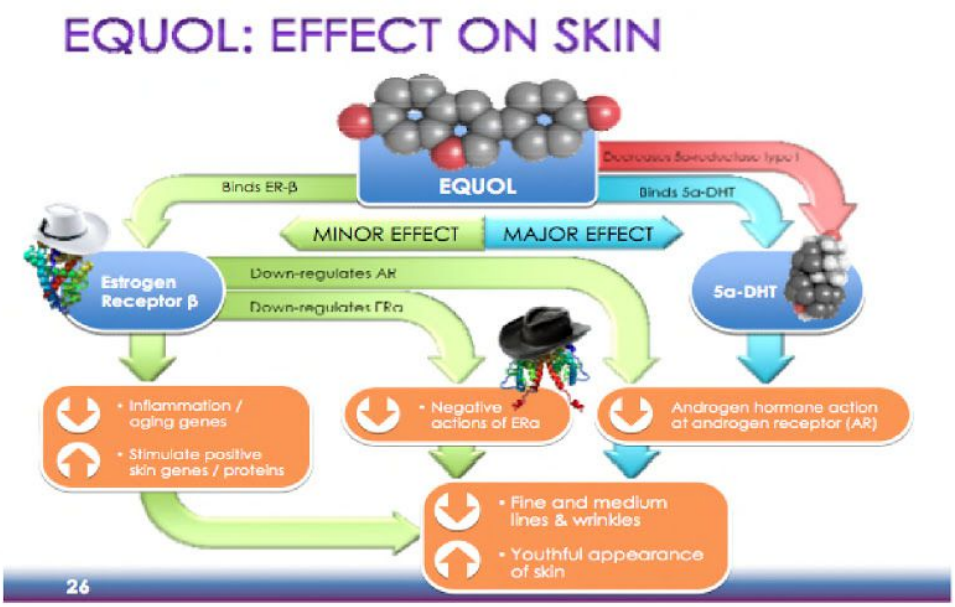

Figure 7. A summary of equol's actions on skin. Equol's major influence is to specifically bind 5α-DHT and significantly decrease 5α-reductase enzyme (type 1), which dramatically reduces androgen hormone actions at the androgen receptor. At the same time equol binds to estrogen receptor β to significantly decrease the expression of inflammation and aging genes, plus it significantly stimulate the expression of positive skin genes like collagen and elastin. Thus, the total effect of equol in skin reduces the appearance of fine and medium lines/wrinkles to restore the youthful appearance of skin.

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