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Research Article

Comparison of Neonatal vs Adult Fibroblast Production of Collagen Type I (*In-vitro*) with 17β-estradiol or Phytoestrogen (Equol) Short-term Exposure

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Abstract

Introduction: Established differences between young versus adult dermal fibroblasts characteristics and functions have been previously reported, which are due to various changes in skin cells with aging such as cellular senescence, etc. However, a comparison of human neonatal versus adult dermal fibroblasts has not been investigated in the production of collagen type I along with testing estrogenic stimulatory compounds like phytoestrogens (equol and its isomers) or the primary natural steroid hormone, 17β-estradiol in short-term cultures.

Methods: Experiment 1 tested neonatal human dermal fibroblasts exposed to R-equol, racemic equol, S-equol or 17β-estradiol at 10 nM for 48 hours. Experiment 2 tested adult skin cells in tri-culture (epidermal keratinocytes; dermal fibroblasts and epidermal melanocytes in a 1:1:1 ratio) exposed to R-equol, racemic equol, S-equol or 17β-estradiol at 10 nM or 50 nM for 48 hours.

Results: Collagen type I levels in neonatal fibroblasts (in controls and under estrogenic stimulation) displayed a significantly greater robust response compared to the adult tri-cultured cells with the same estrogenic treatments. In general, the neonatal cells compared to adult cells showed more than a seven-fold increase in controls levels and more than an eight-fold increase in the various treatments, however, there were no significant differences among the treatments.

Conclusion: The findings of this study should encourage investigators to clearly state the age of human fibroblasts that are utilized in experimental reports, since evidently the results are dependent upon the age of the donor tissue/cells or age range of the derived human dermal cells.

Keywords: Fibroblasts; Neonatal; Adult; *In-vitro*; Collagen; Estrogenic Exposure

Introduction

Established differences between young versus adult dermal fibroblast characteristics and functions have been reported. These include, decreased collagen production in chronological aged skin along with slower migration in adult than neonatal cells, plus changes in cell senescence with aging is known to increase collagen degradation by Matrix Metalloproteinases (MMPs) and also, the expression of growth factors, deposition of extracellular matrix constituents like hyaluronic acid and the physical-mechanical strain or the deformation of dermal fibroblasts all influence collagen synthesis [1-6]. Additionally, hormone chemical messengers such as estrogens or estrogenic compounds via estrogen receptors have been shown to stimulate collagen type I in dermal fibroblasts in cell culture [1,7].

Previous reports using neonatal human dermal fibroblasts in culture demonstrated that short-term exposure to 17β-estradiol and/or the polyphenolic/isoflavonoid compound, equol, significantly increased collagen type I levels in a robust manner [8,9].

Interestingly, equol is expressed as two isomers, R-equol and S-equol or when synthesized in equal concentrations it is known as racemic equol [9]. Each isomeric form of equol has differential positive influences on collagen type I production and the expression of skin-related genes as reported in microarray analysis studies [9]. Plus, the affinity for estrogen receptors by 17β-estradiol and equol have been reported with implications to the predominant expression of Estrogen Receptor Beta (ERβ) in human skin [1,7,10]. These findings provided motivation to investigate whether there are differences in neonatal vs. adult dermal fibroblasts (*in-vitro*) production of collagen type I when exposed short-term to 17β-estradiol or the equol isomers.

Thus, the primary purpose of this study was to determine whether neonatal vs. adult dermal fibroblasts respond in different ways to 17β-estradiol or the equol isomers in the production of collagen type I in short-term cell cultures. In addition, secondary endpoints were examined in experiments with adult dermal fibroblasts, adult epidermal keratinocytes and epidermal melanocytes that were tri-cultured to represent potential interactions such as influencing the behavior of and/or the possibly of affecting the collagen production by dermal fibroblasts. Finally, in the adult cell culture experiment two physiological concentrations (at 10 nM or 50 nM) of 17β-estradiol, R-equol, Racemic equol or S-equol were tested to determine cell viability, collagen type I levels in ng/ml and potential differential influences of the treatments.

Methodology

Neonatal Human Monolayer Dermal Fibroblasts (short-term) Culture Analysis of Type I Collagen and Toxicity (Glo MT Cell Viability) with Equol or 17β-Estradiol Treatments

The goal of experiment 1 was to quantify the protein expression of human type I collagen from primary monolayer dermal fibroblasts via *in-vitro* cultures as previously reported [8,9]. In brief, this was performed by using fibroblasts from neonatal foreskin [in DMEM (MediaTech Inc., Manassas, VA, USA); with 1x non-essential amino acids (HyClone, Logan, UT, USA); 1x antibiotic/antimycotic (Sigma Chemical Co., St. Louis, MO, USA) and 2% bovine calf serum (Invitrogen Corp., Carlsbad, CA, USA) as reported previously [8,9]. Approximately 5e5 cells per 10 cm dish were cultured for 24 h in a 37◦C humidified incubator with 5% CO_2, then the medium was changed, the test materials were dissolved in [dimethyl sulfoxide (DMSO, Sigma Chemical Company, St. Louis, MO, USA). 17β-estradiol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Racemic equol was purchased from LC Laboratories (Woburn, MA, USA) and R- and S-equol were purchased from R Handa at Colorado State University (Fort Collins, CO, USA). Thus, R-equol, racemic equol, S-equol or 17β-estradiol in Dimethyl sulfoxide (DMSO) (all treatment levels were at 10 nM; n = 4 per treatment group) were added and cultured for 48 h. The amount of the type I collagen deposited was quantified by Enzyme-Linked Immunosorbent Assay (ELISA) using goat anti-collagen I capture antibody (cat.# 1310-01; Southern Biotechnology, Birmingham, AL, USA) for capture, biotinyalted anti-collagen I antibody (cat.# 1310-08; Southern Biotechnology) streptavidin-HRP and TMB reagents for detection, sandwich ELISA protocol and was precisely quantified using purified collagen standards with a standard curve (R2 = 0.9875) (Southern Biotechnology) at 450 nm abrorbance. Untreated controls were also included as previously reported [8,9]. Finally, RealTime-Glo MT cell viability assay from Promega (Madison, WI, USA) detected the reducing potential of viable cells using a non-lytic luciferase reaction and thus proportional to the metabolic activity of these cells, which is a substantial improvement over the MTT method, in terms of sensitivity and percision.

Adult Human Epidermal Keratinocytes, Dermal Fibroblasts and Epidermal Melanocytes (Short-Term) Culture Analysis of Type I Collagen and Toxicity (Glo MT Cell Viability) With Equol or 17β-Estradiol Treatments

The goal of experiment 2 was to quantify the protein expression of human type I collagen from adult primary tri-cultured epidermal keratinocytes, dermal fibroblasts and epidermal melanocytes at a ratio of 1:1:1. In brief, this was performed using adult human epidermal keratinocytes (HEK, Cell Applications, San Diego, CA, USA, cat.# 102-05a, passage 3-times), adult human dermal fibroblasts (HDF, Cell Applications, cat.# 106-051, passage 4-times) and adult epidermal melanocytes (HEM, ATCC, Manassas, VA, USA, passage 5-times) in a specialized co-culture medium from Zen-Bio (Durhan, NC, USA, cat.# Cnt-PR-CC) following the protocol outlined above (for deposition of cells per dish and culture conditions). The test treatments were prepared in DMSO at 100 nM and further dilutions were made in distilled sterile water immediately before the start of the experiment. Final treatment doses were 10 nM and 50 nM (n = 5) and incubated for 48 hours as previously described above for experiment 1. At the end of the experiment (t = 48 hours), type I collagen was quantified in the cell culture medium by ELISA and cell viability was determined by the RealTime-Glo protocol (see above).

Lightfield Micrographs of Adult Human Epidermal Keratinocytes (HEK), Dermal Fibrobasts (HDF) and Epidermal Melanocytes (HEM)
Lightfield micrographs of each adult cell type (HEK, HDF and HEM) in culture of control representative samples were performed using 60x or 50x BioQuant® Image Analysis software (Nashville, TN, USA).

Statistical Analysis
All data sets were tested by one-way analysis of variance (Kruskal Wallis) followed by pairwise comparisons (Bonferroni) to determine significance among the various treatment groups. This was accomplished by using SPSS software as the statistical program. All the data were expressed as the mean ± standard deviation of four independent samples by treatment in experiment 1, five independent samples in experiment 2 and $p < 0.05$ was considered significant.

Results

The Equol and 17β-Estradiol Treatments (at 10 nM) Increased the Expression of Collagen Type I in Neonatal Human Dermal Fibroblasts After 48-hour Incubation
As shown in Fig. 1, collagen type I expression in the controls was 225 + 15 ng/ml. All treatments were 10 nM, R-equol increased collagen levels to 350 + 35 ng/ml, racemic equol displayed collagen levels at 369 + 38 ng/ml, while S-equol levels were at 342 + 28 ng/ml compared to 17β-estradiol at 325 + 26 ng/ml. However, while all treatments significantly increased collagen levels compared to control levels, there were no significant differences between or among the treatment values. The top of Fig. 1 displays the cell viability values where controls were at 100 % followed by all the treatment levels that ranged from 92 to 95 percent.

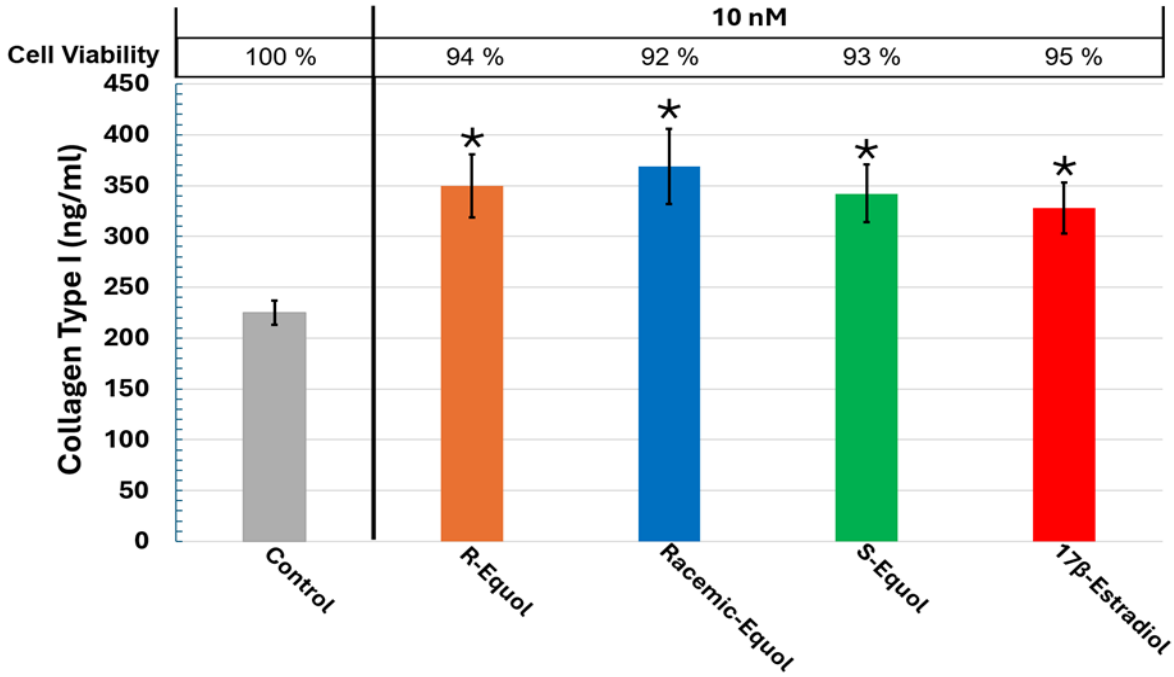

Figure 1: Collagen type I expression in neonatal human dermal monolayer fibroblasts in short-term (48 h) cultures via R-equol, racemic equol, S-equol and 17β-estradiol at 10 nM stimulation. *Indicates significantly greater collagen type I expression compared to untreated control levels. Collagen was quantified by ELISA, n = 4 per treatment group. The collagen data were expressed as the mean + standard error of the mean (SEM). Cell viability at the top of this figure quantified via RealTime-Glo MT among the treatments ranged from 92 to 100 percent, which were not significantly different from each other.

The Equol and 17β-Estradiol Treatments (at 10 nM and 50 nM) Increased the Expression of Collagen Type I in Adult Human Dermal Fibroblasts
After 48-hour Incubation in Tri-cultured Cells. As shown in Fig. 2, representative samples of light field photomicrographs in control samples of each cell type (adult epidermal keratinocytes, adult human dermal fibroblasts and adult epidermal melanocytes) used in this experiment are displayed in panels 1 through 3. Notably, there were no significant alterations in cell shape or volume with the treatments.

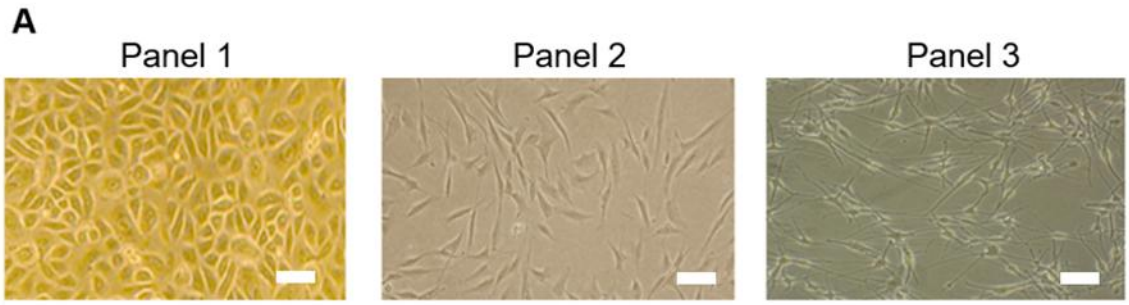

Figure 2: Light field of representative samples shown in: Panel 1- Adult Human Epidermal Keratinocytes (AHEK); Panel 2- Adult Human Dermal Fibroblasts (AHDF); Panel 3- Adult Human Epidermal Melanocytes. White bar = 30 microns.

As displayed in Fig. 3, collagen type I expression in the controls was 29 + 1 ng/ml. For treatments at 10 nM, R-equol increased collagen levels to 33 + 3 ng/ml, racemic equol displayed collagen levels at 39 + 5 ng/ml, while S-equol levels were at 47 + 8 ng/ml compared to 17β-estradiol at 38 + 5 ng/ml. However, while all treatments significantly increased collagen levels compared to control levels, there were no significant differences between or among the treatment values. The top of Fig. 3 displays the cell viability values where controls were at 100 % followed by all the treatment levels that ranged from 98 to 109 percent. However, among the 10 nM treatments for cell viability, racemic equol and S-equol displayed significant cytoprotecting influences in the tri-cultured cells compared R-equol and 17β-estradiol.

For treatments at 50 nM, R-equol increased collagen levels to 44 + 5 ng/ml, racemic equol displayed collagen levels at 37 + 6 ng/ml, while S-equol levels were at 44 + 7 ng/ml compared to 17β-estradiol at 36 + 6 ng/ml. However, while all treatments significantly increased collagen levels compared to control levels, there were no significant differences between or among the treatment values. The top of Fig. 3 displays the cell viability values where controls were at 100 % followed by all the treatment levels that ranged from 98 to 108 percent. But, among the 50 nM treatments for cell viability, 17β-estradiol displayed significant cytoprotecting influences in the tri-cultured cells compared to all other treatments.

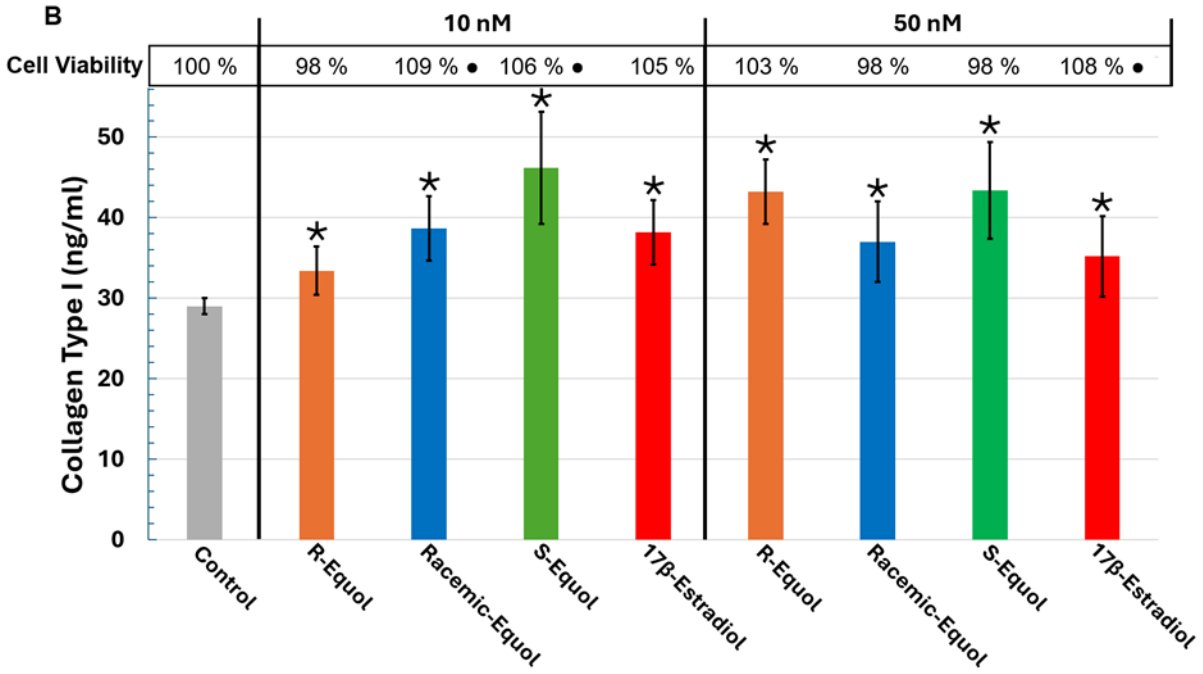

Figure 3: Collagen type I expression in adult human epidermal keratinocytes, dermal fibroblasts and epidermal melanocytes in tri-culture (1:1:1 ratio) in short-term (48 h) exposed to R-equol, racemic equol, S-equol and 17β-estradiol at 10 nM or 50 nM stimulation (n =5 per treatment group). See figure 1 legend for details. ● indicates significant cytoprotecting effects for racemic equol and S-equol at 10 nM and 17β-estradiol at 50 nM compared to the other treatments.

When the magnitude of the neonatal HDF collagen type I expression at 10 nM for the various treatments were compared to the obtained values of the adult HEK, HDF and HEM tri-culture cells at either 10 nM or 50 nM, neonatal cells displayed a more robust and significant response compared to the adult cell cultures. For example, neonatal cells showed more than a seven-fold increase in control levels and more than an eight-fold increase in the various treatments. Conversely, one might suggest that the tri-cultures effectively diluted the HDF by approximately 66 percent compared to the neonatal cultures. Even if this difference was accounted for and adult collagen type I levels were adjusted by this amount, control neonatal collagen values were still 3-fold greater, and the various treatments were more than 5-fold higher in the neonatal vs adult cell cultures. Finally, the cytoprotecting effects by some of the treatments as indexed via cell viability, while significant did not play an important factor in the stimulation of collagen type I, which is noted but marginal in the interpretation of the obtained data.

Discussion

Neonatal fibroblasts and adult fibroblasts exhibit key differences in their collagen type I production as well as other functions [2-6]. In general, neonatal fibroblasts produce more collagen type I compared to adult fibroblasts [2]. For example, in 2006, Varani, et al., reported that young subjects (18-29 years of age) had collagen type I levels 3-times that of older adults (80 plus years of age), which is most likely due to the greater number of senescent cells in aged adults that produce more MMPs that are known to degrade collagen [5]. In fact, cellular senescence plays an important role in skin aging as far as function, dermal health and appearance. Cellular senescence is defined as a state in which cells can no longer proliferate but escape apoptosis despite the accumulation of damage to their DNA and organelles [11]. Senescent cells accumulate in major compartments of skin during aging that secrete pro-inflammatory factors such as cytokines and chemokines, plus proteases, extracellular vesicles, metabolites and lipids that promote cellular/tissue dysfunction that are referred to as a Senescent-Associated Secretory Phenotype (SASP), which cause degradation of the extracellular matrix [11].

In the first experiment of the present study neonatal fibroblasts were utilized, which displayed robust level collagen I in controls and the estrogenic treatments that corresponded to collagen levels from previous reports by our laboratory [8,9]. Conversely, in the second experiment that utilized a tri-culture of adult epidermal keratinocytes, dermal fibroblasts and epidermal melanocytes the collagen I levels in controls and the estrogenic treatments were much lower by approximately 7- to 8-fold compared to the neonatal fibroblasts collagen I results. This may be due to differential collagen I gene expression and functional differences in neonatal vs adult fibroblasts along with neonatal fibroblasts that significantly synthesized more hyaluronic acid that correlates with enhanced migration for collagen deposition [3,12-14].

While adult keratinocytes and melanocytes do not produce collagen I [15], the purpose of the second experiment with tri-cultured adult cells was to determine the potential interactions such as influencing the behavior of and/or the possibly of affecting the collagen production by dermal fibroblasts, which may apply more to wound healing, where keratinocytes may produce collagen as part of their role in re-epithelialization [15,16]. Additionally, keratinocytes have been shown to regulate the turnover of collagen by producing MMP-1 and synthesizing collagen type III, VI and VIII after dermal injury [15,16]. Additionally, melanocytes can produce Melanocyte Stimulating Hormone (α-MSH), which can suppress collagen production in fibroblasts and regulate the production of MMPs to alter collagen deposition, and this may be a factor in the low levels of collagen I observed in experiment 2 [17]. Furthermore, the potential cross-communication between melanocytes and fibroblasts that involve various enzymes, growth factors and signaling molecules might also have played a role in decreased collagen 1 levels recorded between experiment 1 vs experiment 2 in the present study [18]. However, the mechanisms underlying the decline in collagen synthesis with aging have not been fully delineated and further research is warranted.

Moreover, in experiment 2 there were no apparent changes in cellular shape with the estrogenic treatments. However, some reports suggest the estrogens induce a rapid change in cytoskeletal structure via the non-classical receptor GPR 30 [19]. Most likely specific biomarkers are required to detect this alteration [19]. Regarding estrogenic influences on skin, it is known that sun-exposed skin (known as photoaged skin) does not respond as effectively to estrogen treatments as sun-protected skin [20,21].

Strengths and Limitations

This is the first *in-vitro* report and review comparing neonatal dermal fibroblasts to adult fibroblasts for collagen type I expression using physiological estrogenic concentrations. Since cosmetic and dermatological reports use this skin cell type to test active

ingredients the source of the fibroblasts should be stated to help evaluate the obtained results. Conversely, the neonatal vs the adult fibroblast cell characteristics were not the same, which was intentional to represent the differences in the source of fibroblasts by age that may have contributed to the present obtain results. Thus, this report highlights the need for further investigations to determine the differences in fibroblasts characteristics by age.

Conclusion

In summary, to date, this is the first time neonatal dermal fibroblasts have been compared to adult dermal fibroblasts for collagen type I expression. As demonstrated collagen I synthesis in neonatal fibroblasts (in controls and under estrogenic stimulation) displayed a significant greater robust response compared to adult fibroblasts with the same estrogenic treatments. This finding should encourage investigators to clearly state the age human fibroblasts are utilized in experimental reports, since evidently the results are dependent upon the age of the donor tissue/cells or age range of derived human dermal cells.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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None.

Data Availability Statement

Not applicable.

Ethical Statement

The project did not meet the definition of human subject research under the purview of the IRB according to federal regulations, and therefore, was exempt.

Informed Consent Statement

Informed consent was taken for this study.

Authors' Contributions

All authors contributed equally to this paper.

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