A RANDOMIZED PILOT STUDY OF THE RELATIONSHIP BETWEEN ARNOX LEVELS AND THE APPEARANCE OF SKIN AGING IN HEALTHY WOMEN

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INTRODUCTION

The cosmetic industry has a significant focus on preserving youthful appearance in skin. Many products are designed to slow the appearance of aging in the skin because youthful skin is often perceived to be more healthy and attractive than aged skin. The discovery of proteins associated with the aging process can lead to the creation of new cosmetics which inhibit these proteins and thus slow the aging process or the appearance of aging.

An aging related ECTO-NOX protein (arNOX) has been found to function as an NADH (hydroquinone) oxidase. Unlike other ENOX proteins which carry out 4 electron transfers to molecular oxygen and form water, the arNOX proteins result in the generation of superoxide at the cell surface. The superoxides generated are able to form H_2O_2 and other reactive oxygen species which can spread to neighboring cells and tissues. Thus, arNOX is capable of oxidizing hydroquinones of the plasma membrane [1]. As with other ectoproteins, arNOX is not anchored to the membrane and can be shed into the circulation. It can therefore be measured from serum, saliva or perspiration samples. arNOX has been isolated from the serum of older individuals (70-100 years) but less so from the serum of younger individuals (20-40 years). It has been proposed to transmit cell surface oxidative changes to neighboring cells and to be implicated in the development of atherogenesis [2–3].

The purpose of this study was to learn more about arNOX and to determine whether or not arNOX levels are lower in those with healthy and youthful appearing skin compared to those whose skin appears less healthy for their age. This was a pilot study to determine the feasibility of completing a larger and more definitive follow-up study in the future.

A secondary aim was to determine which aspects of skin aging seemed to make the most significant difference in estimating an individual's age. Previous studies have demonstrated that skin tone and color distribution are important components of the perception of skin age [4]. In the current study, the photographs were evaluated for several features to determine the association of these not only with arNOX levels, but also with the estimations of skin age made by the graders.

MATERIALS AND METHODS

Twenty-five female Caucasian subjects between 45 and 65 years of age with no history of cosmetic surgery or current use of cosmetically active ingredients were recruited for participation in the study. The protocol was approved by the Stanford University Research Compliance Office.

At the screening/baseline visit, after informed consent was obtained, each subject was given a skin health and past medical history questionnaire. Each subject had photographs taken of her face from both the front and side with the VISIA® Complexion Analysis System. Subjects were given a glass of water to drink and then 30 minutes later were given a sterile urine cup in which to collect 2 cc' of saliva. Sweat samples were collected through use of a sweat patch which was applied at the clinic visit. Subjects were asked to wear heavy clothing and walk briskly for 20 minutes prior to returning for removal of the patch. Finally, blood was drawn and serum was prepared.

All specimens were sent to Purdue University for estimation of arNOX levels. arNOX activity was assayed according to the following protocol: Measurements of superoxide production were based on the standard method based on reduction of ferricytochrome c by superoxide monitored from the increase in absorbance at 550 nm with reference at 540 nm for 45 minutes [5]. Superoxide dismutase (SOD) was added after 45 minutes to determine if the rate returned to baseline, in order to verify the specificity for arNOX. The assay consisted of 150 μl (2 mg/ml) oxidized ferricytochrome c solution and 200 μl of biofluid added to 2.5 ml PBSG assay buffer (8.06 g NaCl, 0.2 g KCl, 0.18 g Na_2HPO_4, 0.26 g KH_2PO_4, 0.13 g $CaCl_2$, 0.1 g $MgCl_2$, 1.35 g glucose dissolved in 1000 ml deionized water, adjusted to pH 7.4, filtered and stored at 4° C). A SLM Aminco DW-2000 spectrophotometer in the dual wavelength mode with continuous measurements (over 1 minute every 1.5 minutes) was used to determine the rates.

Initially several samples were missing in transport. These were located; however, not until after unblinding. Given the preliminary nature of the study, the decision was made to go forward with analysis and include these values in the study. They were analyzed, then baselines were checked and corrected if needed and outliers were repeated.

Following enrollment of all 25 subjects, 5 independent graders reviewed the photographs taken by the VISIA scanner at the baseline visit and estimated each subject's age. These graders did not know the inclusion/exclusion criteria for the study and thus were not informed of the age range specified by the protocol. Each grader viewed only a close-up of the subject's face so that clothing, hair style and color, etc. were not incorporated into the photograph and assessment. The graders estimated age and, scored skin according to overall skin health, fine wrinkling, deep wrinkling, skin color, skin laxity, pore size and evenness. These estimates of age were averaged and compared to the subject's actual age. The errors in estimating age were calculated as the difference between the mean estimated age and the actual age. Actual age was calculated as the difference between the subject's birthdate and the date of their visit in years including 2 decimal places. The age differences as well as other skin assessment scores were compared to the arNOX levels determined from the serum, saliva, and sweat collections. The graders scoring of overall skin health and specific attributes were compared to their age assessments to determine which characteristics of skin aging correlated most strongly with the age estimates.

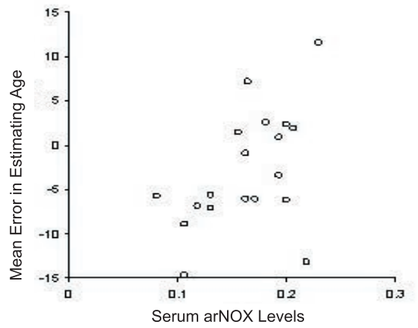
The data were analyzed several different ways in order to give the most accurate portrayal of the relationship between arNOX and age. The data were analyzed without the missing data points, with the located samples included, and with all of the corrected data after outliers were repeated.

RESULTS AND DISCUSSION

Twenty-four subjects' photographs and samples were included in the final analysis. One subject's skin assessment was excluded due to poor image quality.

The initial data analysis included blinded analysis of 19 of the 25 total serum samples. The mean saliva arNOX level was 0.202 with a standard deviation of 0.04. The mean sweat level was 0.198 with a standard deviation of 0.046. The mean serum level was 0.164 with a standard deviation of 0.042. While the means for these levels were similar, the rates did not correlate, suggesting variability in the levels within individual subjects. Saliva arNOX levels and sweat arNOX levels were not correlated with the mean error in age estimation (p=0.77 and p=0.21 respectively); however, serum arNOX levels did correlate with the error in estimating age (p=0.04) (Figure 1).

Figure I: *The mean error in estimating age was positively associated with the serum arNOX levels such that those perceived to be older than their actual age were found to have higher serum arNOX levels than those perceived to be the same age or younger than their actual ages.*



The serum arNOX levels were found to be significantly associated with assessors' measures of fine wrinkles, deep wrinkles, skin laxity score, evenness score, and overall skin health (p<0.05 for all). In this preliminary analysis, there was no association seen between serum arNOX levels and actual age (p=0.779) or subject assessment of skin health (p=0.374) (Table I).

Table I: *Correlations between serum arNOX levels and various attributes of skin quality as rated by independent, blinded graders. N=19*

Attributes of Skin Quality	p value
Actual age	0.779
Subject assessment of overall skin health	0.374
Subject assessment of wrinkling	0.198
Assessors mean fine wrinkle score	0.0292
Assessors mean deep wrinkle score	0.0294
Assessors mean overall skin health score	0.0028
Assessors mean skin color tone score	0.060
Assessors mean skin laxity score	0.0221
Assessors mean pore size score	0.5231
Assessors mean evenness score	0.0263
VISIA score for wrinkles	0.5974

When all 25 samples were located and tested, the data was re-analyzed. Again, no correlation was noted between saliva or sweat samples and age assessments. There was no correlation noted between arNOX levels and actual age (p=0.44); however, there was a significant correlation between arNOX levels and the assessors overall skin health score (p=0.02) and a trend toward correlation with assessors mean deep wrinkle score (p=0.17), mean apparent age (p=0.19) and the mean error in estimating age (p=0.19).

After review of the initial testing, several problems and outliers were noted and these samples were retested. Again, the results were analyzed to determine the impact of the outliers on the overall data set. In this instance, the arNOX levels were found to correlate significantly with the actual patient age (p=0.01), but did not correlate significantly with any of the assessors skin scores.

A second component of the study was the evaluation of the assessors' ability to estimate age and the factors which were most important in their efforts to estimate the age of the subject. Because this portion of the study did not involve the arNOX samples, all 24 subjects were included in the analysis from the outset. Fifteen women were estimated to be older than their calendar age with a mean error of -7.38 years. Nine women were estimated to be younger than their calendar age with a mean error of 6.94 years. Mean scores were calculated for the 5 assessments taken by the independent graders and were compared to the VISIA Complexion Analysis results and to assessments of the subject age. The assessors fine wrinkle score was consistent with that determined by the VISIA® system (p=0.0156). Many of the specific skin features correlated with the assessment of age so that higher scores were seen in conjunction with an older estimation of age. Overall skin health, deep wrinkles, fine wrinkles, skin color tone, skin laxity, darkening under the eyes, and fullness of the face all correlated with the estimated age while pore size, evenness of color, and droop of the eyelids did not (Table II). Interestingly, these correlations were seen only in relationship to the estimated ages and not to the chronological ages, for which only darkening under the eyes was found to have a significant association, even though there was a strong correlation between the age estimations and the actual ages (Table III).

Table II: *Correlations between assessments of select attributes of skin health and estimates of skin age.*

Skin Attributes Assessed by Graders	p value
Deep wrinkles	<0.0001
Fine wrinkles	0.0003
Skin color tone	0.002
Skin laxity	<0.0001
Pore size	0.82
Evenness	0.53
Darkening below eyes	0.06
Drooping of eyelids	0.12
Fullness of the face	<0.0001
Overall skin health	<0.0001

Table III: *Correlations between assessments of select attributes of skin health and actual age of subjects.*

Skin Attributes Assessed by Graders	p value
Deep wrinkles	0.4
Fine wrinkles	0.67
Skin color tone	0.92
Skin laxity	0.21
Pore size	0.11
Evenness	0.82
Darkening below eyes	0.01
Drooping of eyelids	0.62
Fullness of the face	0.47
Overall skin health	0.80

CONCLUSIONS

This pilot feasibility study demonstrated a relationship between arNOX and age and raises many questions about the effect of arNOX on skin aging. Previous data have demonstrated a relationship between arNOX levels and actual age which was not seen in the initial sampling; but was noted after the samples were checked and corrected. This discrepancy appears to arise from the manner of baseline correction, which was the principal difference between the two data sets compared.

This is the first study in which any association between arNOX levels and assessors' skin scores has been noted. The consistency of scoring among assessors and among the different measures of skin health as well as the suggestion that arNOX levels may correlate with assessed skin health is exciting. It is possible that arNOX is a marker which rises as our skin ages, but rises faster in those whose skin appears older than its actual age. It is not clear why these findings were not noted in the final analysis after all samples were located and outliers were corrected. It is possible that in a small pilot study the presence of outliers had a significant impact on the overall results. Future studies with larger sample size, timed sample collection, and fully blinded specimen testing are necessary to further elucidate the relationship between arNOX and skin health.

The secondary aim of the study was to determine which characteristics of skin aging were most important to estimation of age. Many characteristics strongly correlated with assessments of skin age. These include skin changes which are targeted by many cosmetic products. Interestingly, several skin changes, such as pore size, evenness of color and droop of eyelids, which are also frequently altered for cosmetic reasons, did not seem to significantly affect estimation of subject age. Perhaps for those interested in preserving a youthful appearance, these attributes should be less of a concern than skin laxity and wrinkle formation.

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