Reduction in the appearance of facial hyperpigmentation by topical N-acetyl glucosamine

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Summary

Glucosamine has been reported to inhibit melanin production in melanocyte culture. It thus has a potential to reduce hyperpigmentation via topical use. Due to stability limitations of glucosamine, we chose to clinically evaluate the stable derivative N-acetyl glucosamine (NAG). Based on *in vitro* Franz cell testing, NAG is a good skin penetrant. In an 8-week, double-blind, placebo-controlled, left-right randomized, split-face clinical test, topical 2% NAG reduced the appearance of facial hyperpigmentation. In a second clinical study involving the topical combination of 2% NAG with 4% niacinamide, an agent previously shown to be clinically active, the effect on hyperpigmentation was greater. Both of these agents are well tolerated by the skin. This high tolerance coupled with relative ease of formulation and stability in solution make NAG, especially in combination with niacinamide, a suitable cosmetic ingredient for use in skin care products dealing with issues of skin hyperpigmentation.

Keywords: clinical, N-acetyl glucosamine, niacinamide, pigmentation, skin penetration

Introduction

N-acetyl glucosamine (NAG) is an amino sugar that occurs widely in nature and in all human tissues. It is best known for its role as a precursor to hyaluronic acid, a high molecular weight water-binding polymer composed of alternating NAG–glucuronic acid disaccharide units. This polymer serves important structural and hydration roles in extracellular matrix in tissues such as joints and skin,[1–3] both in the epidermis and the dermis.

These data have been shared in part as a poster at the American Academy of Dermatology (AAD) meeting (San Francisco, March 2006).

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Glucosamine itself has also been reported to be effective in reducing production of melanin in melanocytes in culture.[4–10] The mechanism identified is inhibition of glycosylation of tyrosinase, an enzyme central to formation of melanin. In human pigmentation, glycosylation is a required process in the conversion of inactive pro-tyrosinase to active tyrosinase. This mechanism certainly thus has potential to reduce melanin production in human skin. However, no clinical data have apparently been published regarding the effectiveness of topical glucosamine.

While glucosamine is of interest for assessment clinically, it is unstable,[11] thus complicating the preparation of a formulation suitably stable for the duration of a clinical study and raising a concern about its long-term practical utility in topical formulations. Thus, the chemically stable NAG is an alternative material suitable for evaluation.

Another material that has in recent years been shown to be clinically effective in improving the appearance of

facial hyperpigmentation is niacinamide.[12,13] Its mechanism, based on cell culture experiments, is inhibition of transfer of melanin-containing melanosomes from melanocytes to keratinocytes. Since niacinamide and NAG have different mechanisms of action, their combination could be more effective clinically.

This report describes the results of clinical testing of topical NAG alone and in combination with niacinamide in improving the appearance of facial hyperpigmentation.

Materials and methods

Chemicals

N-acetyl-D-glucosamine was obtained from Technical Sourcing International (Missoula, MT). Niacinamide was obtained from DSM Nutritional Products (Parsippany, NJ). $^{3}H_2O$ and ^{14}C niacinamide were obtained from Sigma Radiochemicals (St. Louis, MO), and ^{14}C NAG was obtained from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were USP grade.

Skin penetration

Split thickness Caucasian cadaver skin was obtained from a local medical facility. The skin was cut into appropriately sized sections, and mounted in standard Franz-type diffusion cells (exposed skin surface area of 0.79 cm^2) maintained at 34 °C. The receptors (5 mL) were filled with phosphate-buffered saline (pH 7.4) incorporating 1% Tween 20 and 0.02% sodium azide, and the skin was allowed to equilibrate for 2 h. The cells were randomized to treatment based upon prescreening by $^{3}H_2O$ flux through the mounted skin (150 µL for 5 min). Aliquots of the clinical test formulations were spiked with ^{14}C niacinamide and assayed for total radiolabel in triplicate. Approximately 5 µL formulation ($n = 8$) was applied to the individual cells using a positive displacement pipette. The receptor solution was collected and replaced at 2 and 4 h, with a final collection at the termination of the study (6 h). At the end of 6 h, each skin sample was wiped two times with Whatman filter paper soaked with phosphate-buffered saline/Tween 20 and once with 70%/30% EtOH/deionized water to remove unabsorbed (residual) product, and the stratum corneum/epidermis was then separated from the residual dermis by dissection. Skin fractions were dissolved in 0.75–1.5 mL Soluene-350 (PerkinElmer Life and Bioanalytical Sciences, Boston, MA) at 50 °C overnight, and all receptor collections and wipes and solubilized tissue fractions were counted using Ultima Gold (PerkinElmer). All cocktail solutions were assayed for total radiolabel by liquid scintillation using a preset quench curve (Tri-Carb 2500 TR Liquid Scintillation Analyzer, PerkinElmer).

For data analysis, disintegrations-per-minute (DPM) for each compartment of each cell were blank corrected and summed to obtain a total recovered radiolabel value for a given cell. The DPMs of each compartment were then normalized to the total recovered radiolabel value to obtain a "percentage recovered dose" parameter for each compartment (0–2, 2–4, and 4–6 h receptor collections, stratum corneum, epidermis/dermis, and wipes). In addition, a total skin value was calculated as the sum of the stratum corneum and epidermis/dermis values and a total permeated value calculated as the sum of total skin and cumulative receptor at 6 h.

Clinical testing

Before participating in the two clinical studies described here, each subject signed a written informed consent that contained all the basic elements outlined in 21 Code of Federal Regulations (CFR) 50.25. It explained the type of study, the procedures to be followed, the general nature of the materials being tested, and any known or anticipated adverse reactions that might result from participation. Due to the cosmetic nature of this study, a formal external review of an institutional review board or ethics committee was not done. However, the protocol was reviewed and approved by qualified Procter & Gamble clinical, toxicology, and regulatory personnel and by corresponding personnel at the clinical site. The study was monitored for compliance with the protocol.

Japanese facial study

Healthy Japanese female subjects (ages 25–55; $n = 50$) were enrolled in a 10-week, double-blind, placebo-controlled, split-face study with left–right randomization. This study was conducted in Kobe, Japan, in February–April. All enrolled subjects completed the study. All subjects were selected for inclusion based on the presence of hyperpigmentation (solar lentigo, chloasma, freckles) on both sides of the face.

Prior to the start of the study, there was a 2-week washout period in which subjects were instructed to discontinue use of their normal facial products for the duration of the study. All subjects were given the same set of commercial products to use: facial cleanser, clear lotion, milky lotion, moisturizing cream, and SPF-15 sunscreen. All of these are used in a typical facial treatment regimen used by Japanese women. Subjects were permitted to continue the remainder of their normal facial treatment regimen (e.g., eye and lip makeup and

foundation) that did not contain actives (e.g., α-hydroxy acid, salicylic acid, vitamin A, arbutin). After the 2-week washout period, use of all the supplied products was continued for the remainder of the study, except they were additionally supplied with test formulations (placebo control and the same formulation containing 2% NAG) which were packaged in 30-mL blind-coded pump jars labeled with subject number and the designation of "left" or "right." To the assigned side of the face was applied approximately 0.3 g of formulation (two pumps from the supplied pump jar) of each assigned test formulation, twice daily (morning and evening) for 8 weeks, with the evening application occurring at least 1 h before bedtime. This 0.3 g dosage of test formulation is consistent with the normal facial product use habits of Japanese women. Subjects were supplied with new containers of test formulations at baseline and at week 4 during the 8-week treatment period. Subject compliance with instructions was performed by having subjects complete a daily test formulation use diary, in a return visit to the study site after 1 week of test formulation usage to review the diary and their product use habits, and by weighing the returned test formulation containers (at week 4 and 8). These compliance checks indicated subjects were following test formulation use instructions.

Digital images of each side of the face of all subjects were captured at baseline and at weeks 4 and 8. The images were captured on untreated skin (skin was not treated with test formulations that day) after washing with the assigned facial cleanser commercial product. Before image capture, subjects equilibrated in a controlled temperature room (24 ± 2 °C) for 30 min. Subjects had their hair and clothing covered with black drapes. The images were taken using the same imaging equipment under the same conditions (lighting, distance, head position, etc.) at all time points. The equipment consisted of a high-resolution digital camera (Fuji SC430 CCD Digital Camera, Fuji, Valhalla, NY) equipped with a cross-polarized filter (Tiffen glass polarizing filter, Kenko polarizing filter for light source). Facial illumination was provided by National fluorescence light Twin 1 (FPL27EX-N, 6200–6400°K) positioned to the right and left sides of the camera to provide even lighting. Accurate repositioning of the subjects was facilitated by comparing side by side the live image with the digitally stored image obtained at baseline. The system was calibrated by white balancing the camera each study day. Computer analysis of the digital images allowed quantification of total area of hyperpigmented spots in the selected region of interest (around the cheek to the temple). Facial hyperpigmented spot area on each side of the face was objectively measured using a customized image analysis technique described previously.[12,14]

Caucasian facial study

Healthy Caucasian female subjects (ages 35–65; $n = 35$) were enrolled in a 10-week, double-blind, placebo-controlled, split-face study with left-right randomization. This study was conducted in Cincinnati, Ohio, in October–December. The two test products were 4% niacinamide and 4% niacinamide plus 2% NAG. This study also had additional experimental treatments (additional subjects), which are not a topic of this report. However, these experimental treatments were also paired against the same control (4% niacinamide; additional $n = 70$) and against placebo formulation (no niacinamide; $n = 35$). These were used in the data analyses to increase the statistical power of the study. All enrolled subjects completed the study. All subjects were selected for inclusion based on the presence of hyperpigmentation (solar lentigo) on both sides of the face.

As in the Japanese Facial Study, prior to study start, there was a 2-week washout period in which subjects were instructed to discontinue use of their normal facial products for the duration of the study. All subjects were given the same set of commercial products to use: facial cleanser and facial moisturizer. Subjects were permitted to continue the remainder of their normal facial treatment regimen (e.g., eye and lip makeup and foundation) that did not contain actives (e.g., α-hydroxy acid, salicylic acid, vitamin A). After the 2-week washout period, use of all the supplied cleanser product was continued for the remainder of the study, while the facial moisturizer was replaced with moisturizer test formulations (placebo control, formulation containing 4% niacinamide, and formulation containing 4% niacinamide plus 2% NAG), which were packaged in blind-coded 30-g opaque tubes, labeled "left" or "right." To each side of the face was applied approximately 0.45 g of each assigned test formulation, twice daily (morning and evening) for 8 weeks, with the evening application occurring at least 1 h before bedtime. This 0.45 g dosage of test formulation is consistent with the facial product use habits of the test subjects. Subjects were supplied with new containers of test formulations at baseline and at week 4 during the 8-week treatment phase of the study. Subject compliance with instructions was performed by having subjects complete a daily test formulation use diary, in a return visit to the study site after 1 week of test formulation usage to review the diary and their product use habits, and by weighing the returned test formulation containers (at weeks 4 and 8). These compliance checks indicated subjects were following test formulation use instructions.

All skin measurements were done on untreated skin (skin was not treated with test formulations that day) at

least 30 min after washing with the assigned facial cleanser commercial product. Subjects acclimated their skin in a controlled temperature (21 °C ± 2 °C) and relative humidity (30–50%) room for 30 min prior to measurements (taken under the same temperature and humidity conditions). For imaging, subjects had their hair and clothing covered with black drapes. The images were taken using the same imaging equipment under the same conditions (lighting, distance, head position, etc.) at all time points.

Digital images of each side of the face of all subjects were captured at baseline and at weeks 4 and 8. The facial images were recorded using a Rapid Evaluation of Anti-aging Leads (REAL 2.0) facial imaging system. The REAL 2.0 imaging system captures facial images (wrinkles/fine lines and texture) with a 1.2 mega-pixel digital camera (Fuji HC2500C) equipped with a Fujinon BMD 8*12 with +2 Tiffen close-up lens. A professional grade Balcar flash unit (full spectrum with UV, color temperature 5000 K) is located above the head in a custom portable light box with facial positioning/repositioning capability. Illumination of the face during image capture is done as either sunny day (directional) for texture assessment or diffuse (cloudy day) for color assessment using moveable reflection boards. Each image contained a calibration chart (color chips) used for on-the-fly color correction. This allowed correction for light level and camera response variation using RGB values of the color chart in each image. While the camera and illumination stayed in rigid fixed position, the chin rest was rotated 40° to reposition the subject's head to image capture the right or left side of the face, respectively. After each imaging session, subjects resumed test formulation application.

In the captured images, the facial region of interest for analysis is defined and analyzed to quantify the parameter of interest. Using noncommercial algorithms (developed within Procter & Gamble) for the image analysis, total area of hyperpigmented spots in the selected region of interest (around the cheek to the temple) was determined. The image analysis system has been overviewed previously.[13]

Test formulation effects were also measured by conducting a Visual Perception Study (VPS), wherein expert graders assessed the REAL facial images for several aging skin attributes. Trained graders graded the REAL images. Blind-coded baseline and either 4- or 8-week color images were viewed simultaneously on color-calibrated Barco monitors. The images were identified as "before" and "after," with the "before" images consistently appearing on the left side of the monitor. Graders determined which image looked better for a specific skin attribute and how much better (−8 to +8 scale). Negative numbers indicated that the "after" image was worse than the "before" image. Positive numbers indicated that the "after" image looked better. Zero (0) indicated that there was no difference in the two images. Two graders consensus graded the images.

Clinical study data analyses

In both clinical studies, computer image analyses were done by calculating percentage area fraction = (total hyperpigmented area)/(total skin measurement area) × 100%.

Statistical analysis

For skin penetration experiments, total cumulative receptor, wipes, stratum corneum/epidermis, dermis, total skin, and total permeated values were analyzed using a mixed model including subject (random effect) and products. Receptor values (0–2, 2–4, and 4–6 h) were analyzed separately using the same model. A 10% level of significance was used.

For clinical efficacy variables (image analysis spot area fraction and expert VPS) were analyzed using a mixed model (SAS 8.2 Proc Mixed) for repeated measures with the subject effect fitted as random, and the other effects [treatment, side (left vs. right), time (week 4 vs. week 8), number of spots at enrollment, treatment-by-time interaction] fitted as fixed. Where appropriate, the baseline measurement was added to the model as a covariate. Pairwise differences between adjusted means were considered significant if the *P*-value was less than or equal to 0.05 (two-sided).

Results

Skin penetration

In *in vitro* human skin penetration experimentation, both NAG (Fig. 1) and niacinamide (Fig. 2) in formulation were found to readily penetrate into and through human skin. The former penetrated approximately as well as niacinamide, which has previously been shown to be an effective skin penetrant.[15–17] Also, the presence of niacinamide did not affect the delivery of NAG (Fig. 1), and likewise the presence of NAG did not affect the delivery of niacinamide (Fig. 2).

Japanese facial study

This 8-week, double-blind, split-face clinical study among female Japanese subjects evaluated the effect of topical 2% NAG vs. vehicle control. There was some reduction in spot area in the vehicle control group, an effect likely due to seasonal fading of hyperpigmentation

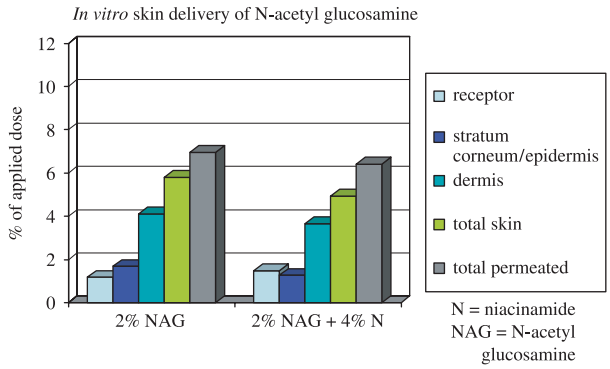

Figure 1 Delivery of N-acetyl glucosamine (NAG) into and through human skin *in vitro*. The topical dosing formulations contained 2% NAG or the combination of 2% NAG + 4% niacinamide (N).

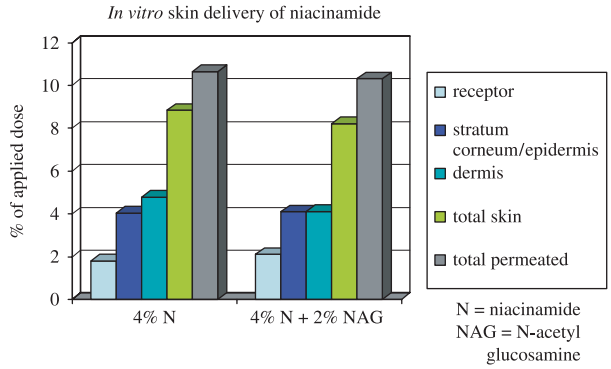

Figure 2 Delivery of niacinamide (N) into and through human skin *in vitro*. The topical dosing formulations contained 4% N or the combination of 4% N + 2% N-acetyl glucosamine (NAG).

in this winter–early spring study. Topical 2% NAG was effective in improving the appearance of facial hyperpigmentation based on computer image analysis, with an overall directional ($P = 0.089$) spot area fraction change across the entire study (combined weeks 4 and 8) (data not shown).

Caucasian facial study

This 8-week, double-blind, split-face clinical study among female Caucasian subjects evaluated the effect of topical 2% NAG in combination with 4% niacinamide vs. niacinamide alone vs. vehicle control. Two objective measures of the appearance of hyperpigmentation were used: computer image analysis for hyperpigmented spot area and expert grader blinded assessment of the captured images. There was substantial reduction in pigmentation in the vehicle control group (Fig. 3), again likely due to seasonal fading of hyperpigmentation in this

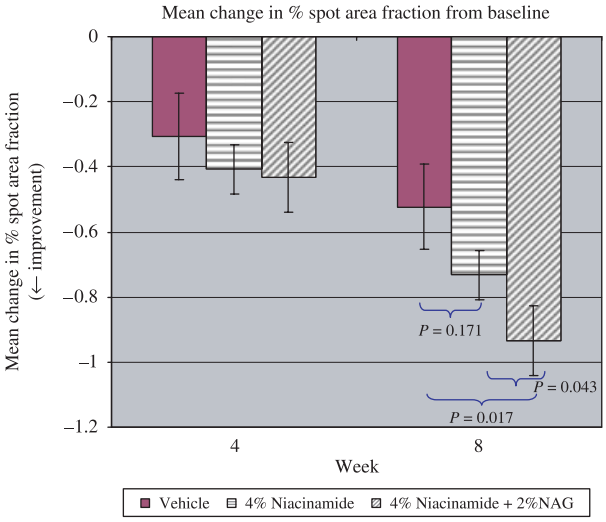

Figure 3 Computer image analysis of Caucasian facial digital images for change in spot area fraction. More negative numbers indicate reduction in hyperpigmentation.

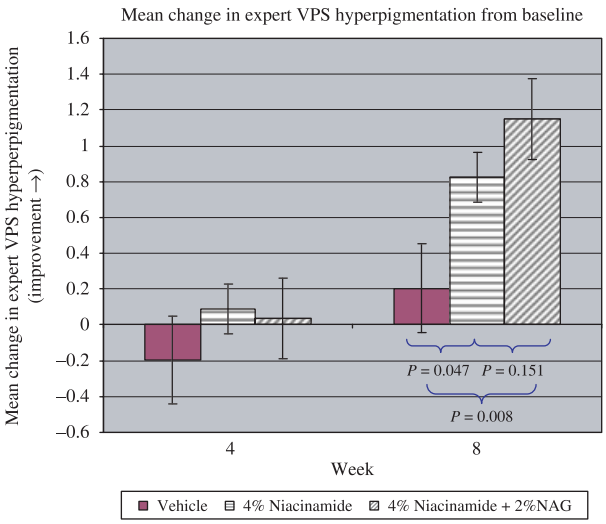

Figure 4 Expert grader assessment of blind-coded Caucasian facial images by Visual Perception System (VPS). More positive numbers indicate less hyperpigmentation.

fall–winter study. Based on both hyperpigmentation measures, at the week 8 time point, topical 2% NAG in combination with 4% niacinamide was more effective than 4% niacinamide which was more effective than the vehicle control (Figs 3 and 4). The greatest significance was observed for the combination vs. vehicle control, with $P = 0.017$ (Fig. 3) and $P = 0.008$ (Fig. 4). In one measure (computer image analysis, Fig. 3), the combination was also significantly different ($P = 0.043$) vs. the 4% niacinamide control, while in the other

A (vehicle)

B (4% niacinamide)

C (4% niacinamide + 2% NAG)

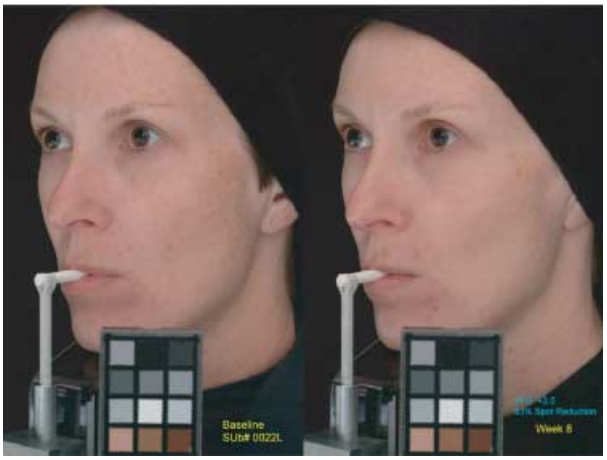

Figure 5 Example images from the 8-week Caucasian facial clinical study. In all image pairs, the before-treatment image is on the left, and the after-treatment image is on the right. A is vehicle control treatment; B is 4% niacinamide treatment; and C is 4% niacinamide + 2% N-acetyl glucosamine treatment.

measure (expert grader, Fig. 4) 4% niacinamide was significantly different from vehicle. Example images from the study are shown in Figure 5.

Discussion

In the relatively small base size clinical testing reported here, topical NAG alone was effective in reducing the appearance of facial hyperpigmentation. Combined with niacinamide, which has previously been shown to be effective in clinical testing,[12,13] the effect was greater. This advantage for the combination appears not to be due to any impact on skin delivery of the materials in the combination. The advantage may derive from inhibiting two different mechanisms of pigmentation. Niacinamide inhibits melanosome transfer.[12] Published *in vitro* work[4–10] describes a mechanism by which glucosamine inhibits melanin production: inhibition of tyrosinase glycosylation. It is likely that NAG also operates via this mechanism, either directly or as the deacetylated species as a result of amidase hydrolysis to free glucosamine. There are also published reports[18–21] describing other mechanisms by which glucosamine compounds provide benefits, such as anti-inflammatory effects. We have in progress *in vitro* experiments to develop further mechanistic information on NAG. Regardless of mechanisms, there is potential for NAG and especially its combination with niacinamide to have utility in improvement of skin hyperpigmentation problems.

The seasonal fading of hyperpigmentation observed here in these fall–winter studies highlights the value of a vehicle control, as opposed to only a baseline control. If only a baseline control had been used, the apparent effects of the treatments would have been greatly exaggerated and clearly not an accurate assessment of the potency of the technologies. It is also likely that studies conducted in spring–summer would encounter increasing hyperpigmentation from seasonal tanning. Those would likewise need to be vehicle-controlled to accurately assess the potency of a tested technology. This anticipated tanning also argues for the inclusion of a sunscreen in such pigmentation testing and as a normal part of daily skin care routine.

N-acetyl glucosamine was well tolerated, from a toxicology standpoint, by facial skin in the clinical testing reported here. Glucosamine compounds in general have been reported to have few if any side effects, even from large oral doses.[22] This high tolerance coupled with relative ease of formulation and stability in solution make NAG, especially in combination with the well-tolerated niacinamide,[23] a suitable cosmetic ingredient for use in skin care products dealing with issues of skin hyperpigmentation.

Glucosamine itself is oxidatively unstable in solution at near-neutral pH, turning yellow-brown (data not shown). That instability led to our choice of NAG, which is stable in solution, as the specific material to evaluate clinically. The dose of 2% was chosen since substantially higher doses (e.g., 5%) are sticky/tacky and thus not aesthetically suitable to ensure clinical subject compliance on the face for the 8-week duration of our testing.

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