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Collagen glycation and skin aging

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Abstract

The Maillard reaction is a non-enzymatic browning process involving reducing sugars and amino groups of amino acids or proteins. It occurs in most foods on heating and also takes place *in-vivo*, in living organisms. At body temperature, this process, called protein glycation, occurs more slowly. But the reaction products accumulate during aging, especially if long-lived proteins, such as structural collagen or lens crystallins, are affected.

Maillard reaction products are irreversible and detrimental for protein function as they lead to protein crosslinking. They have been implicated in pathologies associated with diabetes, atherosclerosis, and Alzheimer's disease. But for all individuals, the consequences of protein glycation are involved in the general aging phenomenon.

Collagens are important proteins for the skin, as they are essential for structure and function of the extracellular matrix in the dermis. Thinner and wrinkled skin, the typical signs of normal aging, are the consequence of reduced collagen. Protein glycation contributes to skin aging as it deteriorates the existing collagen by crosslinking. Accelerated skin aging is especially noticeable in diabetic patients, where glycation is increased because of the high serum glucose level.

For diabetic patients, drugs against glycation are available; but as glycation significantly contributes to skin aging in everybody, we have looked for safe anti-glycation substances as ingredients in cosmetics. Since the formation of dangerous crosslinking glycation products is dependent on oxidation reactions, the application of antioxidants in cosmetic products was the strategy we chose to prevent glycation. As antioxidants, we used a mixture of a water soluble extract from grape seeds and lipid soluble tocopherol. This mixture was tested for inhibition of protein glycation by in vitro glycation assays with the anti-glycation drug, aminoguanidine serving as the control. Our results show that antioxidants do indeed protect against protein glycation to a similar extent as that achieved with aminoguanidine.

Factors that induce skin aging

Cutaneous aging processes can be divided into two groups - intrinsic and extrinsic processes. Extrinsic aging is mainly the result of skin exposure to environmental stresses such as UV-light or pollution (*Scharffetter-Kochanek et al*. 2000).

There are different theories about the origin of intrinsic aging, commonly called the "biological clock". One theory is based on the observation that diploid cells, such as fibroblasts, have a finite life-span in culture (the Hayflick phenomenon). The consequence of this is cellular senescence that leads to altered gene expression and then to degenerative changes in tissues (*Campisi* 1998, *Faragher* 2000). Another intrinsic mechanism that contributes to skin aging, is damage due to free radicals that accumulate during the lifespan of an individual (the Free Radical theory). The theory of glycation (Maillard theory), is today widely recognised as a further general intrinsic aging mechanism, (*Kasper & Funk* 2001, *Reiser* 1998).

Biochemistry of cutaneous aging

Histological analysis of aging skin shows more profound alterations in the dermis than in the overlying epidermis. The dermis is composed of fibrillar collagen bundles and elastic fibres in a complex array of proteoglycans and other extracellular matrix components. Fibroblast cells are embedded within the matrix. The proteins, collagen and elastin, impart strength and resilience to the skin. Histologically, skin aging is associated with a

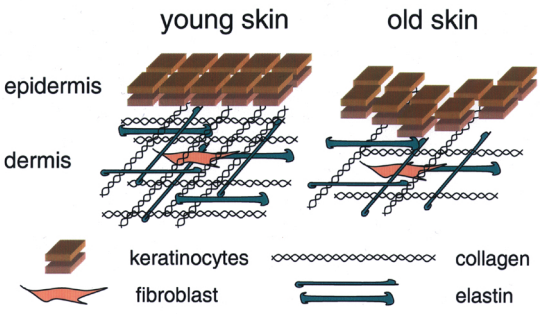

Figure 1. Schematic drawing of young and old skin.

profound atrophy of dermal connective tissue (see **Fig. 1**). Both the Hayflick phenomenon and the Free Radical theory play a documented role in skin aging.

Senescent fibroblasts have a different gene expression pattern to their still-dividing counterparts. Presenescent fibroblasts express low levels of matrix metalloproteases that degrade extracellular matrix proteins like collagen. They also express relatively high levels of the matrix metalloproteinase inhibitors TIMP-1 and TIMP-3 (tissue inhibitors of metalloproteinases 1 and 3). Upon senescence, the expression of matrix metalloproteases increases and the expression of their inhibitors TIMP-1 and TIMP-3 decreases. Thus replicative senescence in dermal fibroblasts results in a switch, from a matrix-producing, to a matrix-degrading, phenotype (*Campisi* 1998).

A progressive rise of oxidative stress, due to reactive oxygen species that are generated through UV-light or are produced intrinsically, changes the pattern of gene expression that results in both aging and inflammation phenotype. The induction of matrix metalloproteinases is the consequence of the activation of the redox-regulated transcription factors, nuclear factor kappa B (NF–κB) and activator protein 1 (AP-1) (*Lavrovsky et al*. 2000, *Bond et al*. 1999, *Saliou et al*. 1999).

Role of protein glycation in skin aging

Collagen and elastin, the two major structural proteins of human tissue, are subjected to molecular changes such as intermolecular cross-linking and side-chain modifications, (*Bailey* 2001). Pyridinoline crosslinks are formed enzymatically by lysyl oxidase. This precise enzymatic process is important for correct development of the extracellular matrix. Nonenzymatically formed cross-links are the result of spontaneous chemical reactions between proteins and sugars. Through Amadori rearrangement, and advanced Maillard reactions, advanced glycation end products (AGE) are formed (see **Fig. 2**). The AGE structures, pentosidine and mold, are protein crosslinkers between lysine and arginine or two lysine residues, respectively.

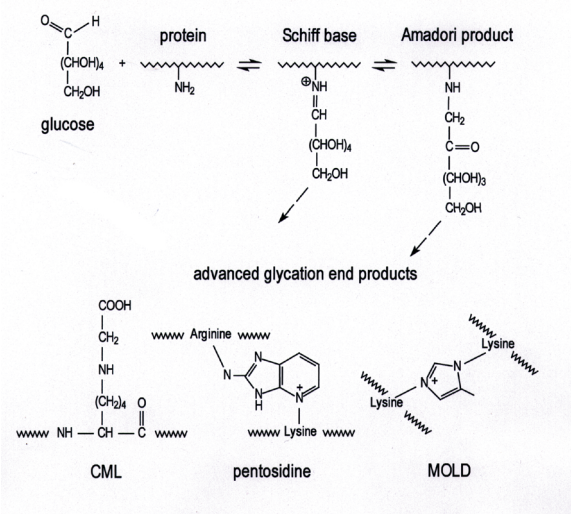

Figure 2. Mechanism of formation of the advanced glycation end products (AGE) pentosidine, carboxymethyllysine (CML) and methyl glyoxal-lysine dimer (MOLD).

Pentosidine was found in increasing amounts with age and diabetes, in plasma proteins, lens crystallins and collagen-rich tissues. Protein glycation as a non-enzymatic process is slow and therefore proteins with a long biological half-life, such as collagen, are more affected. Analysis of Maillard reaction products in the skin collagen of diabetic and nondiabetic control subjects (see **Fig. 3**) showed an age-related accumulation of glycated collagen in both groups (*Dyer et al*. 1993). The initial product in collagen glycation, the Amadori product, was found to be increased by 33% in normal subjects between 20 and 85 years of age. AGE products such as CML and pentosidine increased up to fivefold. In diabetic patients, the value for the Amadori product was threefold and that for AGE products, twofold higher than in normal subjects.

Glycation has several adverse effects on the characteristics of collagen. It has been shown that the accumulation of Maillard reaction products leads to stiffer and more brittle collagen (*Verzijl et al*. 2000). Glycation has been reported to affect the precise aggregation of collagen monomers into fibres (*Guitton et al*. 1981). Glycation not only influences the properties of collagen and of the extracellular matrix, but also affects matrix-cell interactions (for review see *Reiser*). The extracellular matrix modulates

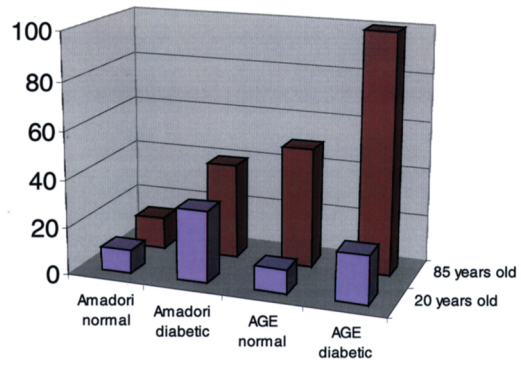

Figure 3. Age-related increase in glycation products (relative units) of skin collagen of normal and diabetic subjects according to Dyer et al. 1993.

many characteristics of resident cells, including migration, growth, proliferation, differentiation, and gene expression. Thus, physical changes in matrix components, such as nonenzymatic glycation of collagen, may affect many cell behaviours. In general it was shown that, cells grown on matrixes composed of glycated proteins differ from cells grown on normal matrix with respect to growth, differentiation, motility, gene expression, and response to cytokines. Several receptors for AGE's have been identified that are expressed on a wide range of cells. This could be a way in which glycated matrix components might influence cell behaviour. The RAGE receptor has been found to accumulate in diabetes, Alzheimer's disease and during aging. Binding of AGE's to the RAGE receptor results in the activation of the NF-κB transcription factor, via generation of oxygen radicals and MAP kinase signalling. NF-κB activation leads to the induction of matrix metalloproteinases and to the formation of pro-inflammatory cytokines (*Singh et al.* 2001).

AGE formation pathways

Protein glycation starts with a nonenzymatic reaction between a sugar aldehyde or ketone, with a free amino group of lysine. The resultant unstable Schiff base product can then undergo an Amadori rearrangement, to a relatively stable Amadori product. Both the Schiff base product and the Amadori product can be transformed by further reactions to AGE's such as carboxymethyllysine (CML), pentosidine or mold. The inert, noncrosslinking CML is a metal ion induced oxidative breakdown product. Pentosidine is the most widely described Maillard structure and has proven to be useful as a protein glycation marker, as it has been found in the collagen of all tissues. Pentosidine is a fluorescent crosslinker, composed of lysine and arginine moieties that are cross-linked by a pentose. As the formation of pentosidine is inhibited in the absence of oxygen, oxidation reactions are required at some stage in its formation (*Baynes* 1991). Meanwhile it seems that there are other mechanisms, besides the Amadori reaction pathway, by which sugars initiate the glycation of proteins, such as the glucose auto-oxidation, the polyol or the triose phosphate-methylglyoxal pathways (*Reiser* 1998). AGE products, that arise by glycooxidative mechanisms, require oxygen and are catalyzed by traces of redox active transition metal ions (*Thorpe & Baynes* 1996).

Strategies to inhibit AGE formation

There are several targets for inhibition of AGE formation. Inhibitors may function as sugar competitors and act by modifying free amino groups of proteins in order to prevent sugar attachment. An example is aspirin, which blocks glycation by acetylating lysine residues. Other inhibitors react with aldose and ketose sugars (protein competitors), diverting them from Maillard reactions with proteins. This class of inhibitors comprises compounds with free amino groups such as the amino acid residues lysine and arginine, and compounds like carnosine or ethanolamine. The best known inhibitor, aminoguanidine, probably acts on more than one step of the Maillard cascade. It reacts with Amadori compounds, but inhibition is thought to act mainly by trapping reactive dicarbonyl intermediates that arise from oxidation reactions of free sugars or Amadori products (*Khalifah et al.* 1999). Aminoguanidine as a hydrazine drug has a negative side effect, because it depletes the body of essential carbonyl compounds such as pyridoxal-5'-phosphate (vitamin B6). Other inhibitors such as pyridoxamine or thiamine pyrophosphate are referred to as post-Amadori inhibitors as they inhibit most effectively at the conversion step of Amadori intermediates to AGEís (*Khalifah et al.* 1999). Since the formation of AGE products is dependent on oxidation reactions, the use of antioxidants like vitamins C and E or the plant cytokin kinetin is another approach to prevent advanced glycation (*Verbeke et al.* 2000).

Protein glycation inhibitors suitable for cosmetics

The use of Aminoguanidine, Pyridoxamine or Aspirin is the pharmaceutical answer that is advised for diabetic patients. For topical application in

cosmetic products, compounds active in the prevention of glycation must be well tolerated, non-irritant and without any toxicity or side effects. Furthermore, the activity must be able to penetrate into the skin, to cross the stratum corneum and finally reach the living parts of the epidermis and the dermis where the detrimental effects of glycation occur.

Monosaccharides, as a source of the glycation problem, should be excluded from cosmetic products. But simple sugars, as essential primary metabolites, are ubiquitous in all living material and are therefore also present in a lot of natural cosmetic ingredients, for example, crude plant extracts or milk fractions. Reducing sugars are often added as a major component in moisturizing products, as humectants, because they are a cheap raw material. In this way glycation substrates are delivered to the skin. Good humectant alternatives would be amino acid residues or lactate, which are not involved in the glycation process.

Self tanning products contain reducing molecules, the most widely used ingredient being dihydroxyacetone. These undergo the Maillard reaction with skin surface proteins to produce a durable brown colour. In the first instance, these molecules react with proteins of the cornified, dead cells of the stratum corneum, cells that are removed with desquamation. A proportion of the ingredients however, will penetrate deeper, reaching living epidermis layers and the dermis, and there promote protein glycation, one of the principal ageing mechanisms.

Because the generation of AGE's is dependent on oxidation reactions, cosmetic products recommended for aging skin should contain antioxidants. In addition, metal chelators should be included, as a proportion of the AGE's are produced by auto-oxidation of glucose where the oxidation is catalyzed by metals (*Thorpe & Baynes* 1996).

Anti-glycation potential of a cosmetic ingredient composed of water and lipid soluble antioxidants.

All living organisms protect themselves with a combination of lipid soluble antioxidants, such as vitamin E (tocopherol) and carotenoids and water-soluble antioxidants, such as vitamin C and glutathione. Grapes, especially the red species such as Pinot Noir, are extraordinarily rich in polyphenols. By far the largest portion is found in grape seeds, in the form of procyanidins. Catechins and epicatechins are the basic units of the procyanidins, which consist of up to 50 monomers. Procyanidins are very powerful water soluble radical scavengers, and are frequently more effective antioxidants than vitamins C or E. For our experiments we isolated procyanidins from grape seeds in a mixture of water, glycerin and alcohol. Vitamin E is naturally present in the skin, where it protects skin lipids against peroxidation. The final cosmetic ingredient that was tested in glycation assays was composed of 85% grape seed extract, 10% solubilizer and 5% alpha, gamma and delta tocopherols (*Zülli et al*. 2001).

Materials and methods

In vitro glycation

Human albumin (Sigma, fraction V) or bovine lens protein (Sigma) was dissolved at 10 mg/ml in 200 mM phosphate buffer, pH 7.4, 500 mM glucose and 0.02% sodium azide and incubated at 37°C. Aminoguanidine hydrochloride (Fluka) was used at a final concentration of 200 mM and the cosmetic antioxidant ingredient at 10%. Samples for analysis of Amadori and AGE reaction products were passed over a PD-10 column, equilibrated in water, to remove Schiff base and free glucose, and stored frozen.

Measurement of Amadori reaction product

Amadori products were measured by the periodate assay, using the protocol developed by *Ahmed and Furth* (1991). The assay is based on quantification of the released formaldehyde after periodate oxidation of the C-1 hydroxyls in the Amadori form of glycated proteins.

Desalted samples (300 µl) were diluted with H2O to 500 µl and incubated with 250 µl 50 mM NaIO4 for 30 min at RT. To terminate the oxidation, samples were cooled on ice for 10 min, and mixed with 250 µl precooled 15 % ZnSO4 and 0.7 M NaOH by vortexing. To remove precipitated zinc periodate, samples were centrifuged for 10 min at 10'000 rpm in an Eppendorf centrifuge. From the supernatants, 500 µl were mixed with 1 ml Formaldehyde detection reagent that was freshly prepared by mixing 46 µl of acetylacetone in 10 ml of 3.3 M ammonium acetate. After 1 h incubation at 37°C, absorbance at 405 nm was measured.

Measurement of AGE products

The formation of AGE's was analyzed by fluorescence measurements of the desalted samples (200 µl) in a microplate fluorescence reader (Bio-Tek, FL 600) at an excitation wavelength of 360 nm and an emission wavelength of 440 nm.

Results

In vitro assays with human serum albumin as the test protein, clearly showed the formation of AGE's in the presence of glucose (**Fig. 4**). Addition of aminoguanidine resulted in 90% inhibition of the formation of glycation end products after 4 weeks. A similar inhibition was achieved with the antioxidant mixture.

Assays with bovine lens proteins showed a similar result, although the inhibition was not as prominent (**Fig. 5**). Aminoguanidine and the antioxidant mixture, reduced formation of glycation end products after 4 weeks by about 40% and 30% respectively.

The same lens protein incubations were assessed for the formation of Amadori products by the periodate assay (**Fig. 6**). Neither aminoguanidine nor the antioxidant mixture showed inhibition of formation of the Amadori product, but rather a stimulation of about 40% after 4 weeks.

Use of aminoguanidine, as well as the application of antioxidants in the prevention of protein glycation, seems to function by blocking the conversion step of Amadori products to AGE's. Greater accumulation of Amadori products in the presence of aminoguanidine or of the antioxidant ingredient, compared to incubation with glucose alone, may be the result of the different kinetic of the overall reaction. When there is a block in the conversion reaction sequence of Amadori product to AGE, the former will accumulate. Only the AGE's are detrimental for the skin, because only these glycation end products cause protein crosslinking and so collagen stiffening.

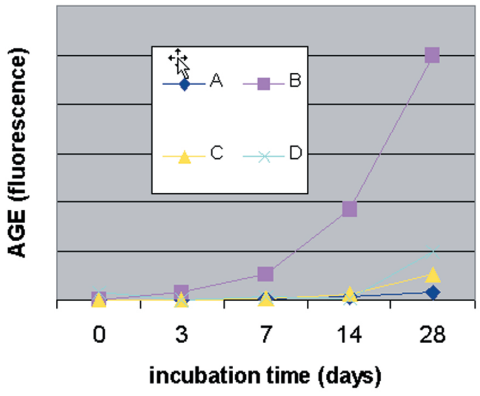

Figure 4. Formation of advanced glycation end products (AGE) with bovine serum albumine (BSA). Formation of AGE's was measured in relative fluorescence units after 3, 7, 14 and 28 days incubation at 37°C. A) control, BSA without glucose, B) BSA with glucose, C) BSA with glucose and aminoguanidine, D) BSA with glucose and antioxidant ingredient.

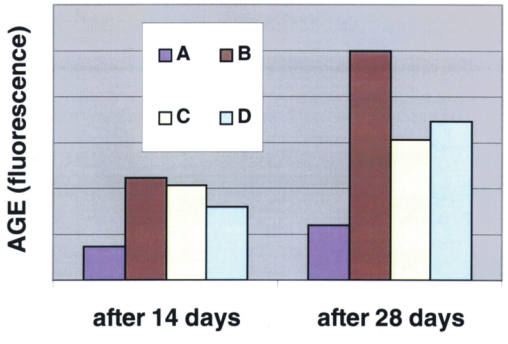

Figure 5. Formation of advanced glycation end products (AGE) with bovine lens protein. Formation of AGE's was measured in relative fluorescence units after 14 and 28 days incubation at 37°C. A) control, lens protein without glucose, B) lens protein with glucose, C) lens protein with glucose and aminoguanidine, D) lens protein with glucose and antioxidant ingredient.

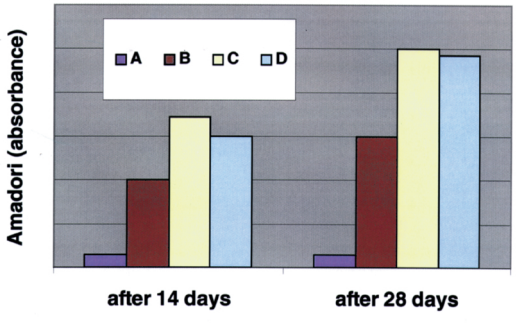

Figure 6. Formation of the Amadori product with bovine lens protein. Formation of the Amadori product was measured in relative absorbance units after 14 and 28 days incubation at 37°C. A) control, lens protein without glucose, B) lens protein with glucose, C) lens protein with glucose and aminoguanidine, D) lens protein with glucose and antioxidant ingredient.

References

Bailey, A. J. (2001) Molecular mechanisms of ageing in connective tissues. *Mechanisms of Ageing and Development* 122: 735-755.

Baynes, J. W. (1991) Role of oxidative stress in development of complications in diabetes. *Diabetes* 40: 405-412.

Bond, M., Baker, A. H. & Newby, A. C. (1999) Nuclear factor (B activity is essential for matrix metalloproteinase-1 and -3 upregulation in rabbit dermal fibroblasts. *Biochem. Biophys. Res. Commun.* 264: 561-567.

Campisi, J. (1998) The Role of Cellular Senescence in Skin Aging. *J. Investig. Dermatol. Symp. Proc.* 3: 1-5.

Dyer, D.G., Dunn, J.A., Thorpe, S. R., Bailie, K. E., Lyons, T. J., McCance, D. R. & Baynes, J. W. (1993) Accum. of Maillard reaction products in skin collagen in diabetes & aging.*J.Clin.Invest*.9:2463-2469.

Faragher,R.G.A. (2000) Aging & the Immune System. Biochemical Society Transactions 28(part 2): 221-226.

Guitton, J. D., Le Pape, A., Sizaret, P. Y. & Muh, J. P. (1981) Effects of in vitro N-glucosylation on type-I collagen fibrillogenesis. *Biosci. Rep.* 12: 945-954.

Kasper, M. & Funk, R. H. W. (2001) Age-related changes in cells and tissues due to advanced glycation end products (AGEs). *Archives of Gerontology and Geriatrics* 32: 233-243.

Khalifah, R. G., Baynes, J. W. & Hudson, B. G. (1999) Amadorins: novel post-Amadori inhibitors of advanced glycation reactions. *Biochem. Biophys. Res. Commun*. 257: 251-258.

Lavrovsky, Y., Chatterjee, B., Clark, R. A. & Roy, A. K. (2000) Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Experimental Gerontology* 35: 521-532.

Reiser,K.M.(1998)Nonenzymatic glycation of collagen in aging & diabetes.*Proc. Soc.Exp.Biol.Med*. 218: 23-37.

Saliou, C., Kitazawa, M., McLaughlin, L., Yang, J.-P., Lodge, J. K., Tetsuka, T., Iwasaki, K., Cillard, J., Okamoto, T. & Packer, L. (1999) Antioxidants modulate acute solar ultraviolet radiation-induced NF-kappa-B activation in a human keratinocyte cell line. *Free Radic. Biol. Med*. 26: 174-183.

Scharffetter-Kochanek, K., Brenneisen, P., Wenk, J., Herrmann,G.,Ma, W.,Kuhr,L.,Meewes,C.& Wlaschek, M. (2000) Photoaging of the skin from phenotype to mechanisms. *Experimental Gerontology* 35: 307-316.

Singh, R., Barden, A., Mori, T. & Beilin, L. (2001) Advanced glycation end-products: a review. *Diabetologia* 44: 129-146.

Thorpe, S. R. & Baynes, J. W. (1996) Role of the Maillard reaction in diabetes mellitus and diseases of aging. *Drugs Aging* 9: 69-77.

Verbeke, P., Siboska, G. E., Clark, B. F. C. & Rattan, S. I. S. (2000) Kinetin inhibits protein oxidation and glycoxidation in vitro. *Biochem. Biophys. Res. Commun*. 276: 1265-1270.

Verzijl, N., DeGroot, J., Oldehinkel, E., Bank, R. A., Thorpe, S. R., Baynes, J. W., Bayliss, M. T., Bijlsma, J. W. J., Lafeber, F. P. J. G. & Tekoppele, J. M. (2000) Age-related accumulation of Maillard reaction products in human articular cartilage collagen. *Biochem. J*. 350: 381-387.

Zülli,F.,Belser,E.,Neuenschwander,M.,Muggli,R. (2001) Antioxidants - grape seeds protect hair against reactive oxygen species.*PersonalCare,October*: 65-67.

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