

Full Length Research Paper

Effect of vitamin C on collagen biosynthesis and degree of birefringence in polarization sensitive optical coherence tomography (PS-OCT)

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Nearly one third of protein in the body consists of collagen. It plays a key role in providing the structural scaffolding for cells, tissues, and organs. With available literature, we show that prolonged exposure of cultures of human connective-tissue cells to ascorbate (vitamin C) induces an eight-fold increase in the synthesis of collagen with no increase in the rate of synthesis of other proteins. Making advantage of increased degree of polarization due to enhanced rate of collagen biosynthesis in the presence of vitamin C, we propose a better imaging modality for diagnosing thermal damage in human tissues.

Key words: Collagen, vitamin C, triple helix, birefringence, optical coherence tomography.

INTRODUCTION

Skin is made up of three layers viz., the epidermis, the dermis and the fat layer. The dermis composed largely of the protein collagen. Most of the collagen is organized in bundles running horizontally through the dermis, which are buried in a jelly-like material called the ground substance. Collagen accounts for up to 75% of the weight of the dermis, and is responsible for the smoothness and elasticity of the skin. It plays a key role in providing the structural scaffolding for cells, tissues, and organs. Major structural protein in human body is collagen which composes of three protein chains wound together in a tight triple helix (Rich and Crick, 1955; Berisio et al., 2002). This unique structure gives collagen a greater tensile strength than steel. A sketch of collagen in human skin is depicted in Figure 1.

Nearly 33% of the protein in the body consists of collagen. This protein supports tissues and organs and connects these structures to bones. Vitamin C induces lipid peroxidation and reactive aldehydes, a step required for collagen expression, collagen m-RNA levels, and coll-

agen production in cultured human fibroblasts. Collagen gene expression is probably influenced by lipid-Perodixation, or through acetaldehyde formation, which consequently increases collagen gene transcription in cultured human fibroblasts (Walingo, 2005). The mesh-like collagen network binds cells together and provides the supportive framework or environment in which cells develop and function, and tissues and bones heal (Lodish et al., 2003). The breakdown of healthy collagen and the decline in collagen production leads to the development of unwanted wrinkles and the appearance of aged skin.

The collagen bundles are held together by elastic fibres running through the dermis. These are made of a protein called elastin (Gigante et al., 2005). As skin gets older, it loses some of its elasticity and ability to retain water. Collagen production declines as does subcutaneous fat, and the facial muscles start to atrophy. Both collagen and elastin fibres are made by cells called fibroblasts, which are scattered through the dermis.

A macromolecule in the ground substance, glycoprotein can hold large amounts of water, and are responsible for maintaining a required amount of water in the dermis. Hyaluronic acid is another important substance that forms part of the tissue that surrounds the collagen and elastin fibres. It has the ability to attract and bind hundreds of times its weight in water. In this way it acts as a natural

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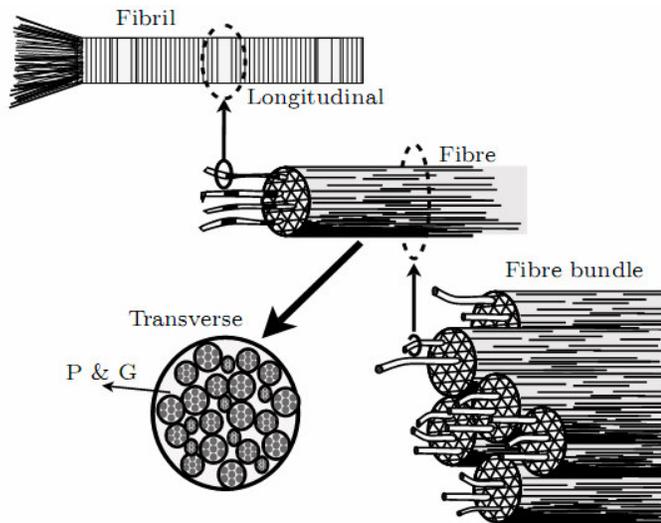


Figure 1. Schematic of Collagen fibril, collagen fibre and primary fibre bundles within a fibre. Proteoglycans and glycosaminoglycans surround the collagen fibres.

moisturizing ingredient responsible for the plumpness of skin and moisture reserve. As one gets older the amount of hyaluronic acid produced in the skin naturally reduces. This is one reason why aging skin becomes less resilient and supple. The hierarchical structure of collagen as shown in Figure 1 reveals the picture of amino acid sequence formation of tropocollagen molecules to collagen fibres.

The triple helix nature of collagen makes it structurally non-centrosymmetric (NCS). In turn it makes it optically birefringent. However, when the collagen is denatured by thermal damage it coagulates and loses the NCS nature and becomes non-birefringent. We make use of this property and progress to adopt it for the diagnosis of thermal damage in tissue using optical coherence tomography (OCT). In addition, possibility of increase in signal to noise ratio (SNR) with the help of vitamin C is also explored. We also predict that with the administration of vitamin C an increase in biosynthesis of collagen will lead to an increase in the degree of birefringence which can be detected by Polarization sensitive OCT.

HYPOTHESIS

Collagen biosynthesis

The main steps in collagen biosynthesis are shown in Figure 2. Intracellular events involve protein synthesis on endoplasmic reticulum to form three polypeptide chains, which make up procollagen; procollagen is hydroxylated to form procollagen, which is glycosylated at the endoplasmic reticulum, degree of hydroxylation of the lysine residue is important in that it will determine the number of cross links and therefore strength of the collagen; procollagen is converted to a triple helix before secretion from cell by microtubules, procollagen helices are actually longer than collagen molecules owing to amino-terminal and carboxy-

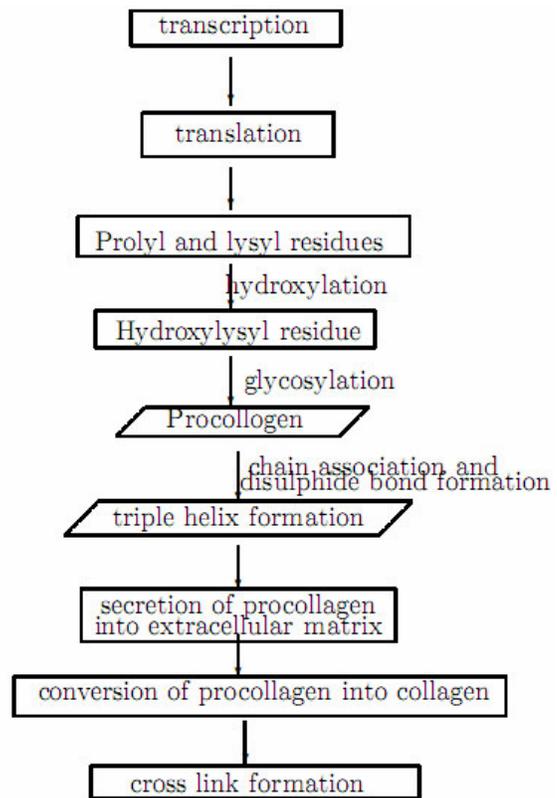


Figure 2. Flowchart of collagen biosynthesis (for details see text).

terminal end pieces that keep collagen soluble while inside the cell; intracellular processes require more than eight enzymes, result in over 150 modifications of each chain, and alter one-tenth of the amino acids. Collagen is created by fibroblasts, which are specialized skin cells located in the dermis. Fibroblasts also produce other skin structural proteins such as elastin (a protein which gives the skin its ability to snap back) and glucosaminoglycans (GAGs). GAGs make up the ground substance that keeps the dermis hydrated. When the receptors are bound by the correct combination of signal molecules (called fibroblast growth factors, or FGFs), the fibroblast begins the production of collagen. Fibroblasts initially produce short collagen subunits called procollagen (Church et al., 1971).

These are transported out of the fibroblast cells and later join together to form the complete collagen molecule. Vitamin C acts as a cofactor during many steps of the process. Without sufficient levels of vitamin C, collagen formation is disrupted. This disruption leads to a variety of disorders such as scurvy - a disease where the body cannot produce collagen and as a result, essentially falls apart as these support structures (collagen) deteriorate.

Collagen protein requires vitamin C for hydroxylation, a process that allows the molecule to achieve the best configuration and prevents collagen from becoming weak and susceptible to damage (Boyer et al., 1998). There is also evidence that vitamin C increases the level of procollagen messenger RNA. Collagen subunits are formed within the fibroblasts as procollagen which are excreted into extracellular spaces. Vitamin C is required to export the procollagen molecules out of the cell. Collagen synthesis occurs continuously throughout our lives to repair and replace damaged collagen tissue or build new cellular structures. The degradation and recycling of old or damaged collagen is a healthy, natural process used to create protein fragments needed to build new cellular

structures, such as in the healing process. With age, collagen levels drop off due to a decrease in production and an increase in degradation (Patricia, 2005).

Promoting the synthesis of new collagen

There are many ways to promote the synthesis of new, healthy collagen: (i) Provide the skin with a reserve of vitamin C. As a necessary cofactor in collagen synthesis, vitamin C is proved to increase the production of collagen. It is shown that extended exposure of human connective-tissue cells to vitamin C stimulates an eight-fold increase in the synthesis of collagen (Silva et al., 2000). (ii) Use of chemical exfoliants, such as alpha-hydroxy and polyhydroxy acids, which break down the bonds between cells of the stratum corneum and slough away dead skin. Consistent exfoliation stimulates cell renewal. Chemical exfoliation has also been shown to increase dermal thickness. (iii) Supplement with collagen stimulating peptides. Fibroblasts are naturally stimulated to begin the synthesis of collagen when specific combinations of peptide signal molecules (fibroblast growth factors) bind to receptor sites on the fibroblast membrane. These signal molecules can be supplemented with topically and help boost collagen production. This paper focuses on the first mode of collagen synthesis promotion by treatment with vitamin C.

Collagen has unusual amino acid composition. It contains large amounts of glycine and proline, as well as two amino acids that are not inserted directly by ribosomes- hydroxyproline and hydroxylysine. The former compose rather a larger percentage of total amino acids. They are derived from proline and lysine in enzymatic processes of post translational modification for which vitamin C is required (Oikarinen et al., 1976). This is related to why vitamin C deficiencies may cause scurvy, a disease that can lead to loss of teeth and easy bruising caused by reduction in strength of connective tissue due to lack of collagen or defective collagen. Collagen fibres consist of globular units of the collagen, sub-units of tropocollagen. Tropocollagen subunits spontaneously arrange themselves under physiological conditions into staggered array structure stabilized by numerous hydrogen bonds and covalent bonds. Tropocollagen subunits are left handed triple helices where each strand is further right handed helix itself. Hydroxylysine and hydroxyproline play important role in the stabilization of the tropocollagen globular structure as well as the final fibre shaped structure by forming covalent bonds. The resulting structure is called collagen helix. Collagen protein requires vitamin C for hydroxylation a process that allows the molecule to achieve the best configuration and prevents collagen from becoming weak and susceptible to damage. First, a three dimensional stranded structure is assembled, with the amino acids glycine and proline as its principal components. This is not yet collagen but its precursor, procollagen. Previously it was shown that vitamin C must have an important role in its synthesis (Nusgens et al., 2002). Prolonged exposure of cultures of human connective-tissue cells to ascorbate induced an eight-fold increase in the synthesis of collagen with no increase in the rate of synthesis of other proteins. Since the production of procollagen must precede the production of collagen, vitamin C must have a role in this step, the formation of the polypeptide chains of procollagen, along with its better understood role in the conversion of procollagen to collagen (Humbert et al., 2001). The conversion involves a reaction that substitutes a hydroxyl group (OH), for a hydrogen atom (H), in the proline residues at certain points in the polypeptide chains, converting those residues to hydroxyproline. This hydroxylation reaction secures the chains in the triple helix of collagen. The hydroxylation of the residues of the amino acid lysine, transforming them to hydroxylysine, is then needed to permit the cross-linking of the triple helices into the fibres and networks of the tissues.

Relation of change in birefringence with polarization

Birefringence in skin is due to the asymmetrical collagen fibril structure and may change if the underlying structure is disrupted due to local heat generation by fire or acid burns (Thomsen et al., 1991; Maitland et al., 1994). The induced heat will change the conformation of collagen from coil form to random coils and the collagen will coagulate (Pearce et al., 1993). The collagen molecule is physically anisotropic. This gives collagen-rich tissues interesting optical properties such as strong linear birefringence and a high non-linear susceptibility. These optical properties can be very well studied by Polarization sensitive Optical coherence tomography.

Polarization-sensitive optical coherence tomography combines polarimetry with low coherence reflectometry to provide high-resolution optically sectioned images of the birefringence of intact tissues (Giattina et al., 2006). Optical coherence tomography (OCT) is an interferometric, noninvasive, noncontact imaging technology that can image biological tissues at micrometer-scale resolutions. OCT detects the interference signal between the back-reflected probe light and the reference light passing through an interferometer. The depth resolution of OCT (1 to 10 μ m) is determined by the source coherence length. Interference occurs only when the optical path-length difference between the probe beam and reference beam is within the coherence length of the broadband light source. The lateral resolution, as in the case of confocal microscopy, is determined by the diameter of the focused probe beam in the sample. The imaging depth of the technique is limited to the quasi-ballistic regime (1 to 2 mm) in scattering biological tissues (Schmitt, 1999). Polarization sensitive (PS) OCT is a recent extension of classical OCT that measures and images birefringence properties of a sample. Light as a transverse wave has polarization, a property we can leverage to enhance contrast via PS-OCT (Schmitt, 1999). This technique is important because polarization exists in many biological components such as collagen, retinal tissue, keratin, and myelin. Furthermore, highly birefringent collagen is ubiquitous in biological tissues, and its birefringence can be altered by denaturalization processes; therefore, these intrinsic polarization contrast mechanisms can be used as diagnostic indicators.

In PS-OCT imaging tissue with organized collagen changes the polarization state of the incident light due to refractive index mismatch between collagen fibre bundles and surrounding fluids, these all contribute to the tissue birefringence. Collagen has two indices of refraction, n_o and n_e , the difference indicating the strength of birefringence. When linearly polarized light passes through a birefringent tissue, it is resolved into two rays, an ordinary and an extraordinary ray. These pass through the tissue at different velocities, resulting in a phase difference, or retardation of one ray relative to the other. Degree of birefringence depends on the thickness of the birefringent material. Once the collagen protein is damaged it cannot be returned to its original form (de Boer et al., 1998). Now emerges, the need for biosynthesis of collagen. From the previous sections it is evident that vitamin C has proved to be fruitful in biosynthesis of collagen. Linearly polarized light that illuminates skin is backscattered by superficial layers and rapidly depolarized by birefringent collagen fibres. It is possible to distinguish such superficially backscattered light from the total diffusely reflected light that is dominated by light penetrating deeply into the dermis (Schoenenberger et al., 1998). By coagulation it is clear that the collagen protein is no more functional, rather it has been destroyed. Since it is already known that its increase is depended on the asymmetrical structure of collagen, its destruction will obviously lead to a decrease in the degree of birefringence. The change in birefringence affects the polarization state of the incident light (Hee et al., 1992). A typical PS-OCT setup is shown in Figure 3. The intensity of the horizontal and vertical components can be given as (de Boer et al., 1998):

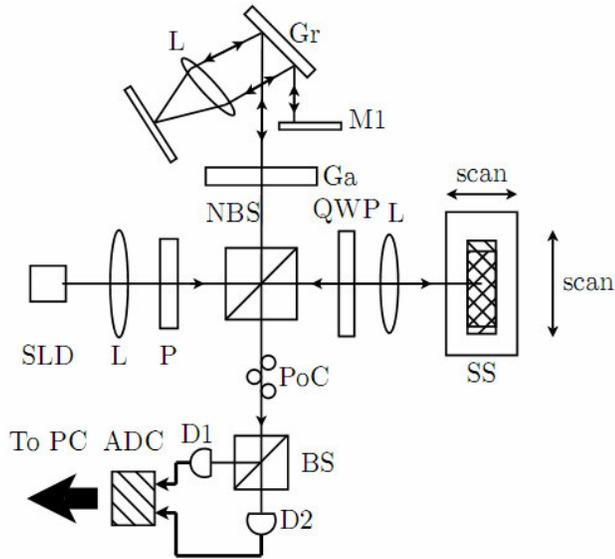


Figure 3. Schematic of proposed experimental setup for PS-OCT. SLD, Superluminescent diode; L, lens; P, polariser; NBS, non polarizing beam splitter cube; Ga, galvanometer; Gr, diffraction grating; QWP, quarter wave plate; SS, motor controlled stages; PoC, fibre optic polarization control; BS, polarizing beam splitter cube; D, photo diodes; ADC, transimpedance amplifier and ADC converter. It measures the two orthogonal components of the electric field vector of the electromagnetic waves using individual photo detectors.

$$I_H(z) = R_H(z) \sin^2(k_o z \delta) \quad (1)$$

and

$$I_V(z) = R_V(z) \cos^2(k_o z \delta), \quad (2)$$

where $I_H(z)$ and $I_V(z)$ are the horizontal and vertical backscattered intensities as a function of depth z . $R_H(z)$ and $R_V(z)$ are reflectivities at z . $k_o = 2\pi/\lambda$ is the attenuation of the coherent beam by scattering. $\delta = (n_s - n_f)$ is the birefringence given by the difference in the refractive indices along fast and slow axes of the sample. The degree of polarization may be obtained from

$$\Phi = \arctan \sqrt{I_H(z)/I_V(z)} = k_o z \delta. \quad (3)$$

The two detectors simultaneously record the signals from orthogonal polarization states of the emerging light which allows complete characterization of tissue via the depth resolved Muller matrix and Stokes parameters (de Boer et al., 1999; de Boer et al., 1997; Jiao et al., 2000). Change in polarization can be calculated using Muller matrix, Stokes parameters and Poincarés Sphere (Jiao et al., 2003).

The state of polarization represented by the polarization ellipse may be described mathematically by the four so-called Stokes parameters. Stokes parameter images of light backscattered from skin will significantly change due to any change in the conformation of collagen by any means as this will lead to change in birefringence.

$$S = \begin{pmatrix} I \\ Q \\ U \\ V \end{pmatrix} = \begin{pmatrix} a_x^2 + a_y^2 \\ a_x^2 - a_y^2 \\ 2a_x a_y \cos \phi \\ 2a_x a_y \sin \phi \end{pmatrix} \quad (4)$$

Where I, Q, U and V are Stokes parameters, $a_{x,y}$ are amplitudes of two orthogonal components of the electric vector and ϕ represents the phase difference between two components. The effect of an optical device on the polarization of light can be characterized by a 4×4 Muller matrix. The matrix acts on the input state S_1 to give the output state S_2 .

$$S_2 = \begin{pmatrix} I_2 \\ Q_2 \\ U_2 \\ V_2 \end{pmatrix} = M S_1, \quad (5)$$

where M is the Muller matrix and $S_1 = (I_1 \ Q_1 \ U_1 \ V_1)^T$. The Poincarés sphere representation provides a convenient method to evaluate changes of Stokes vectors. The horizontal and vertical polarized components of interference intensity between light in the sample and reference arms were detected separately. From these two quantities, the Stokes parameters in the scan can be calculated (Srinivas et al., 2004).

RESULTS AND DISCUSSION

Recently, the ratio of rate of administration of Vitamin C to the rate of production of Placebo an experiment was studied on a population of 10 subjects. (Placebo means an inactive substance or preparation used as a control in an experiment or test to determine the effectiveness of a medicinal drug.) The subjects were treated with w/o emulsion containing 5% L- ascorbic acid (vitamin C) on the external arm [A] and femw/o emulsion (Placebo) [P] on the external face of the other arm, daily for 6 months and the number of copies per unit of mRNA was examined. The studied population is heterogeneous in terms of steady state level of m-RNAs. Only a comparative analysis of the active [A] on one side, placebo [P] on the other side in the same subject can reveal significant differences.

On considering only α 1III collagen the result was as follows: Here we take A/P ratio as the measure of mRNA levels after being treated by vitamin C. Here A tells us about the amount of mRNA for collagen in patients treated with vitamin C and P (Placebo), the amount of mRNA for collagen in patients who have not been administered vitamin C. The third column in Table 1, indicates the ratio of expected change in the degree of polarization in the presence (Φ_c) and absence (Φ_0) of vitamin C administration.

Figure 4 depicts that increase in the levels of mRNA for collagen with linear increase in the percent variation of

Table 1. Results of A/P and corresponding change of degree of polarization.

Tester	$\alpha 1$ III Collagen (A/P)	$(\Phi_c/\Phi_0) \times 100$
1.	1.34	134
2.	1.64	164
3.	0.91	91
4.	1.56	156
5.	1.59	159
6.	0.96	96
7.	1.24	124
8.	0.62	62
9.	0.78	78
10.	1.45	145

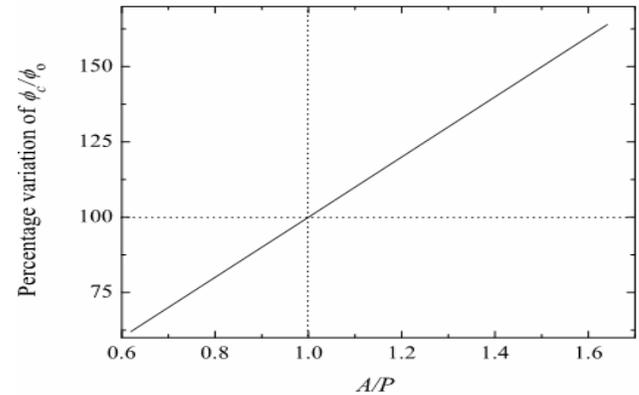
degree of phase retardation. From equation 3 it is clear that the phase retardation has a linear relation with the birefringence δ . From the work Nusgens et al. (2002) it has been proved that with the administration of vitamin C there is an increased synthesis of collagen and with increase in collagen a corresponding increase in birefringence. We can draw a conclusion that with the administration of vitamin C there will be an increase in the level of mRNA for collagen and thus there is obvious increase in the birefringence, which will enable PS-OCT to diagnose the natural and thermally denatured tissues more carefully. Accordingly, Vitamin C can be used in the diagnosis of burn healing as it enhances the biosynthesis of collagen. We can employ PS-OCT to measure the change in birefringence. As vitamin C is involved in the biosynthesis of collagen there will be increase in the asymmetrical nature of collagen which will lead to increase in the birefringence. Again stokes parameter images of light backscattered from skin will significantly change due to any change in the conformation of collagen by any means as this will lead to change in birefringence.

Conclusion

We conclude that birefringence may be a key for diagnosing thermal damage in tissues when used with polarization sensitive optical coherence tomography. The origin of birefringence lies in the biosynthesis of collagen. Further, the diagnosing capability of PS-OCT may be improved eight-fold, if vitamin C is administered, which in turn increases the biosynthesis of collagen.

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**Figure 4.** Percentage variation of degree of polarization in the presence and absence of vitamin C with ratio of A/P. Linear increase in Φ_c/Φ_0 suggests a better signal-to-noise ratio when used with PS-OCT.

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