Chapter

Vitamin C Promotes Wound Healing: The Use of in Vitro Scratch Assays to Assess Re-Epithelialization

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Abstract

Vitamin C contributes to the health of the cardiovascular, immunological and nervous system and also supports healthy bone, lung, and skin function and wound healing. The mechanism of action of vitamin C in human health is as diverse as its targets and effects. For example, vitamin C functions as an antioxidant, signals changes in gene expression, plays a role in protein metabolism, and serves a cofactor in several biosynthetic pathways including collagen synthesis. Here in this chapter we will explore the role of vitamin C in support of improved wound healing during the re-epithelialization stage. While vitamin C supports wound healing in early stages by reducing inflammation, vitamin C continues to support wound healing by promoting collagen synthesis and epithelial cell migration in the re-epithelialization stage. The re-epithelialization stage of wound healing has been modeled and investigated using an in vitro scratch-assay in which a monolayer of epithelial cells is scratched to create a gap or void in the monolayer to represent the wound. The rate of epithelial cell migration back across this gap to re-establish the monolayer can then be used as a model and measurement of the re-epithelialization stage of wound healing. Again, this Chapter will review the literature on both a) the uses of in vitro scratch assays to investigate the mechanism of vitamin C enhanced epithelial cell migration and b) the potential uses of the in vitro scratch assay to study the bioavailability and absorption of liposomal vitamin C.

Keywords: ascorbic acid, PureWay-C®, vitamin C, wound healing, scratch assay, liposomal vitamin C, epithelial cell migration

1. Introduction

Dietary intake of vitamin C is essential for human health and is a nutrient required in the function of a wide range of tissues including the nervous, immune and cardiovascular systems [1–3]. Vitamin C also limits oxidative stress and reduces the risk of cancer [1, 2]. Vitamin C is also essential to skin health and wound healing [1, 2]. While the health benefits of vitamin C are far reaching in the body, the

dietary requirement for vitamin C in human health was first noted by Lind in 1953 [4] in the human disease Scurvy which results in bleeding gums and loosened teeth as well as other signs of blood vessel damage [1, 2, 4]. Vitamin C functions to maintain healthy gums and blood vessels is by acting as a cofactor for the enzymes, prolyl 3-hydroxylase, prolyl 4-hydroxylase, and lysyl hydroxylase which are required for collagen synthesis and assembly in the extracellular matrix [5–7]. Failure to assemble collagens in tissue matrices leads to leaky blood vessels and wounds that cannot heal [2, 4]. Vitamin C also serves as a cofactor for additional enzymes including dopamine β-monooxygenase in the synthesis of norepinephrin [8] and asparaginyl hydroxylase which regulates hypoxia inducible factor (HIF) [9, 10]. HIF is a transcription factor associated with cell transformation and cancer and prolyl 4-hydroxylase, also regulates HIF hydroxylation [11–13]. Another potential cancer fighting characteristic of vitamin C is antioxidant activity in which vitamin C protects human lymphocytes from hydrogen peroxide induced oxidative DNA damage [14]. The antioxidant activity of vitamin C has also been associated with benefits to the human cardiovascular system through low density lipoprotein oxidation and through the quenching of peroxyl radicals and lipid peroxidation metabolism in the blood [1, 2, 15, 16]. As a reducing agent, vitamin C also plays a role in human health through the facilitation of iron absorption in the small intestine [17]. As noted above, vitamin C is important in neurotransmitter synthesis [8] but also has a much broader role in the health of the nervous system [18]. Specifically, in vitro, vitamin C has been shown to stimulate neurite outgrowth in several cell lines including adult hippocampal cell cultures [18] Further, in human clinical studies vitamin C has been shown to reduce the activity of neurotoxins and neurodegenerative diseases and alleviate mood disorders including anxiety and depression [18]. Vitamin C may also plays a role in protection from infection and supporting immune system function [19]. Vitamin C deficiency is historically associated with pneumonias [19, 20]. Consequently the ability of vitamin C supplementation to ward off infections has been widely investigated and includes prevention of sepsis which is likely due to reduced oxidative stress and associated tissue damage and inflammation [21]. Along these lines, most recently a plethora of literature has shown that vitamin C supplementation can help mitigate CoViD-19 associated inflammation [22–26]. However it is interesting to note that the mechanism of action of vitamin C to reduce CoVid-19 and other infection-associated inflammation, may be through more immunomodulatory mechanisms rather than through reduction of tissue stress and damage associated with infection like that see in sepsis and septic shock syndrome [27]. Indeed, vitamin C has direct effects on the cells and signaling molecules of the immune system, promoting T-cell maturation, phagocytic activity in neutrophils, and cytokine and interferon production [19, 20, 28–31]. For example, vitamin C inhibits lipopolysaccharide (LPS)-induced secretion of the proinflammatory cytokine TNF-α and interferon-γ and promotes the release of the anti-inflammatory cytokine IL-10 from lymphocytes [29–31]. Vitamin C is also essential to skin health and wound healing [32, 33]. Vitamin C has been shown to not only to support increased collagen synthesis in skin [34–36], but also to reduce the signs of aging and increase the rate of wound healing [32, 37–40]. Wound healing in the skin involves three stages which are inflammation, re-epithelialization and tissue remodeling [41]. Both the re-epithelialization and tissue remodeling stages of healing wounds involves new collagen synthesis and deposition [42–46] can be supported by vitamin C supplementation. Here in this chapter we review studies using an in vitro scratch assay to study the value of vitamin C supplementation on the re-epithelialization stage of wound healing.

2. Vitamin C in wound healing

Wound healing involves at least three stages, each of which can be influenced by vitamin C to enhance the healing process and reduce tissue damage and scaring [33, 41, 47]. Injuries that breach of epithelial layers represent actual and potential sites of exposure and infection. Consequently there is an immediate inflammatory response to tissue damage and wounds. Inflammation is the first stage of wound healing and involves blood vessel dilation, increased vascular permeability and recruitment of leukocytes to the site of tissue damage. The edema and swelling of inflammation serves as a barrier to microbes from gaining access to the circulatory system and the recruitment of leukocytes to help with any infection and also for autophagy of damaged tissue [48]. While inflammation is an important process and the first stage of wound healing, hyper inflammation at sites of tissue damage can interfere with proper healing and can increase scaring. Indeed vitamin C has been shown to reduce expression of the inflammatory cytokines IL-1β and TNF-α at the wound site in vivo [47]. While vitamin C contributes to wound healing through multiple mechanisms, including through the regulation of inflammatory cytokine production [33, 47], it is interesting to note that nearly all stages of wound healing involves collagen synthesis [41, 48–51]. While the early inflammation stage involves collagen III synthesis, as epithelial cells begin to migrate to close the wound and the underlying connective tissue is re-established, collagen III synthesis must switch to collagen I synthesis so that the epithelial cells can migrate [41]. After the wound closes and re-epithelialization takes place, then basement membrane collagen V and others must be made to finalize the tissue remolding associated with repair [41]. Here, we review the use of the in vitro scratch-assay to investigate the ability of ascorbic acid and various forms of vitamin C to stimulate and promote the re-epithelialization stage of wound healing.

2.1 Vitamin C in scratch assays

The re-epithelialization stage of wound healing has been modeled in vitro using many epithelial cells lines [52–56]. The use of epithelial cells in this wound healing model known as the scratch assay makes it fitting to study the re-epithelialization of wounds [52, 53]. In this system, epithelial cells are grown to complete confluence in test wells. The monolayer of the cells can them be physically damaged or scratched to create a void or gap where the cells have been removed [52–56]. The migration of the epithelial cells back into the void to close the gap models the epithelial migration that takes place during re-epithelialization [52–56]. Using this model of wound re-epithelialization, the effects of drugs, metabolic agents and nutrients on wound healing have been investigated. For example, epidermal growth factor and vascular endothelial cell growth factor have been shown to enhance wound healing in the in vitro scratch assay [53], both of which are also known to support wound healing in vivo [57, 58]. Further, plant extracts from *centell asiatica* (gotu kola) and *crocus sativus* (saffron) have been shown to increase wound healing in the scratch assay and also promote wound healing in vivo [59–61]. With regard to plant phytochemicals and nutrients, vitamin C has also been shown to promote wound healing in scratch assays [62–64]; (**Figure 1**) and also in vivo [32, 37–40]. The photomicrographs of the scratch assays shown in **Figure 1** are 786-0 human epithelial cells over a 24 hour period of wound healing and show that vitamin C increases wound healing in the in vitro scratch assay which has been previously described using a variety of cell

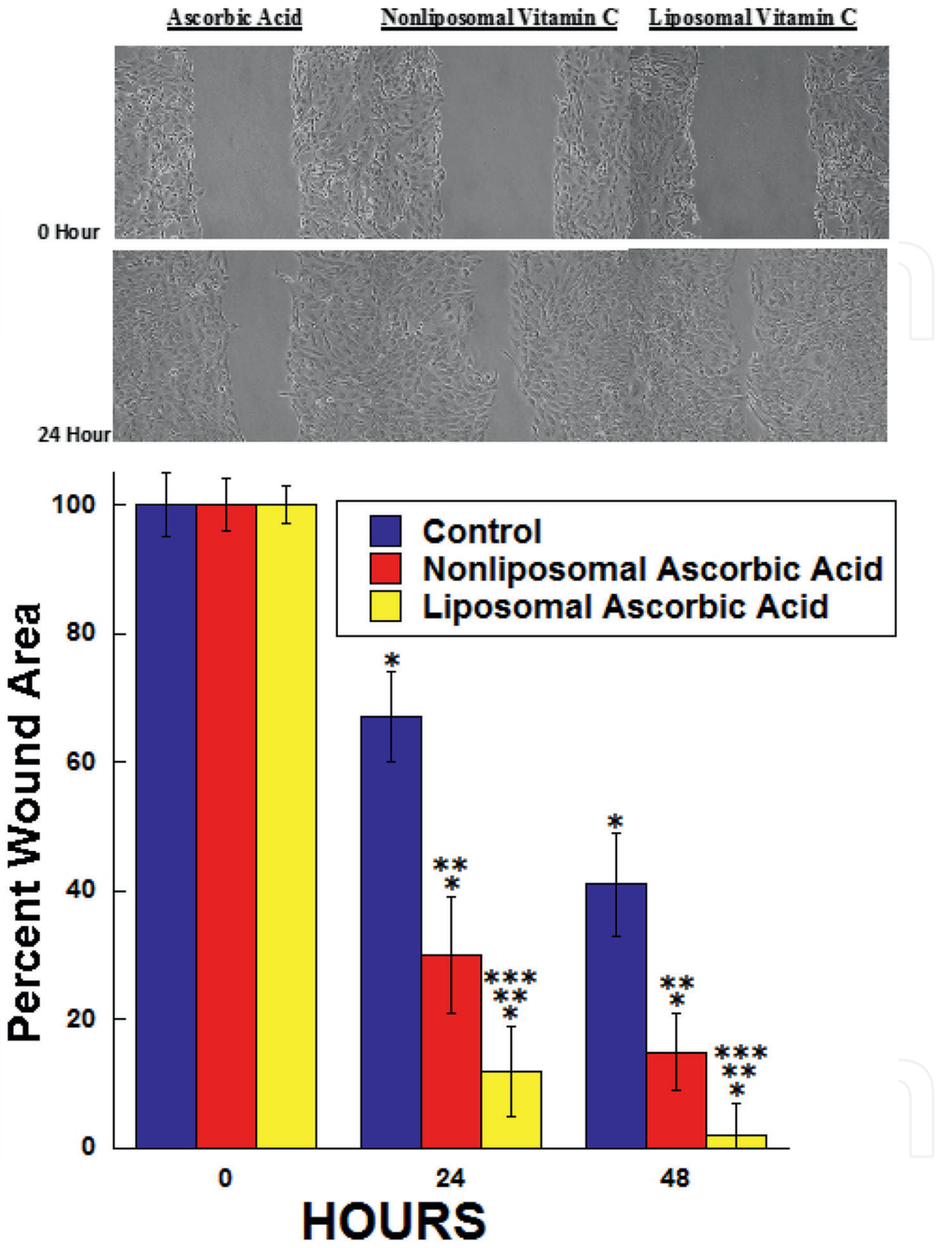

Figure 1.
Top panel: human kidney epithelial cells (786-0) were grown to 100% confluence to produce a monolayer. A wound in the monolayer was produced using a sterile 1000 µl micropipette tip and the cells were then immediately treated with 5 mM ascorbic acid, nonliposomal vitamin C, or PureWay-C® liposomal vitamin C (liposomal vitamin C) and incubated for 24 hours at 37°C in a CO_2 water-jacketed incubator. The monolayers were then photographed at the wound site immediately after treatment and at 24 and 48 hours. The above picture are representative images taken at time 0 (top row) and 24 hours (bottom row). Graph: The size of the wound was determined at time 0, 24 hours and 48 hours by analyzing the images at 0, 24 and 24 hours with imageJ. The wound area at time 0 is considered 100% and the percent wound area was determined for all treatments and time points. In an ANOVA analysis, all 24 and 48 hour treatments showed significant reduction in wound size from the previous day at 95% confidence (*). Further, both nonliposomal and liposomal vitamin C treatment lead to a significant increase of closure at 95% confidence (**). PureWay-C® liposomal vitamin C was significantly more effective than nonliposomal vitamin C at stimulating wound closure at 24 and 48 hours at 95% confidence (***).

lines [62–64]. Photographs of the cells migrating back into the void were analyzed by ImageJ and the area of the void was determined and presented as percent of scratched area (**Figure 1**). With no treatment, the scratch area was reduced by 33% of the original area after 24 hours and 41% after 48 (**Figure 1**). Both a nonliposomal form and a liposomal form of vitamin C reduced the wounded area to greater extent than a control treatment. The nonliposomal form of vitamin C reduced the wound area by 70% and 85% after 24 and 48 hour respectively while the liposomal form (PureWay-C® Liposomal C) reduced the wound area by 88% and 98% after 24 and 48 hours respectively (**Figure 1**). Vitamin C enhanced wound healing involves fibroblast induced collagen synthesis [65], the ability of vitamin C to stimulate collagen synthesis in epithelial cells [66, 67] also plays an important role in wound healing [68, 69] particularly in the production of types IV and V collagens found in the basement membrane of the skin [49, 70]. Therefore, the ability of vitamin C to promote wound healing in the in vitro scratch assay is most likely due to enhanced collagen deposition providing the epithelial cells a substrate to migrate on and close the void created by the scratch.

2.2 Cellular absorption of vitamin C

The contribution of dietary vitamin C to wound healing depends on the absorption of vitamin C in the gut and also into the epithelial and other skin cells at the wound site. Cellular absorption in the gut is regulated by a set of sodium dependant channel proteins known as sodium dependant vitamin C transporters (SVCTs) [2]. This family of proteins is made up of subtypes that are differentially distributed in tissues and accounts for the various different levels of vitamin C absorbed into different tissues [2]. For example, the liver may have as little as 1 mM vitamin C, while the brain and activated leukocytes have as much a 10 mM vitamin C [2, 71]. Both topical application [72–74] and dietary supplementation [32, 75–77] have been used with success to boost vitamin C at the wound site and enhance wound healing [32, 72–77]. Chemical and physical modification of vitamin C has been shown to improve bioactivity and cellular absorption. For example, Ester-C® has been shown to be better absorbed than unmodified vitamin C in leukocytes after oral consumption [78], however in other studies a lipid metabolite extracted for of vitamin C, known as PureWay-C®, was shows to have more bioactivity than Ester-C® [79] and be better absorbed in human leukocytes and into plasma [80, 81]. More recent work has focused on incorporating vitamin C into liposomes [82]. When packaged into liposomes, vitamin C is better absorbed into cells [82–85]; (**Figure 2**) and shows better bioavailability. For example, when compared to nonliposomal vitamin C, vitamin C incorporated into liposomes and administered orally has been shown to within one hour reach significantly higher blood plasma concentrations and plasma taken from these people showed improved protection from lipid peroxidation [83]. In addition, liposomal vitamin C supplementation has been shown to have cardiovascular benefits by reduced hypertension and the risk of ischemia to a greater extent than nonliposomal vitamin C [81–85]. **Figure 2** shows that PureWay-C® liposomal vitamin C is also better absorbed in human epithelial cells than nonliposomal vitamin C. At 10, 30, 60 and 120 minutes liposomal C is better absorbed in epithelial cells by 233%, 180%, 160% and 174% respectively compared to nonliposomal vitamin C. The better absorption of liposomal vitamin C leads to improved collagen synthesis and explains the improved wound healing seen in the literature and exampled here in **Figure 1**.

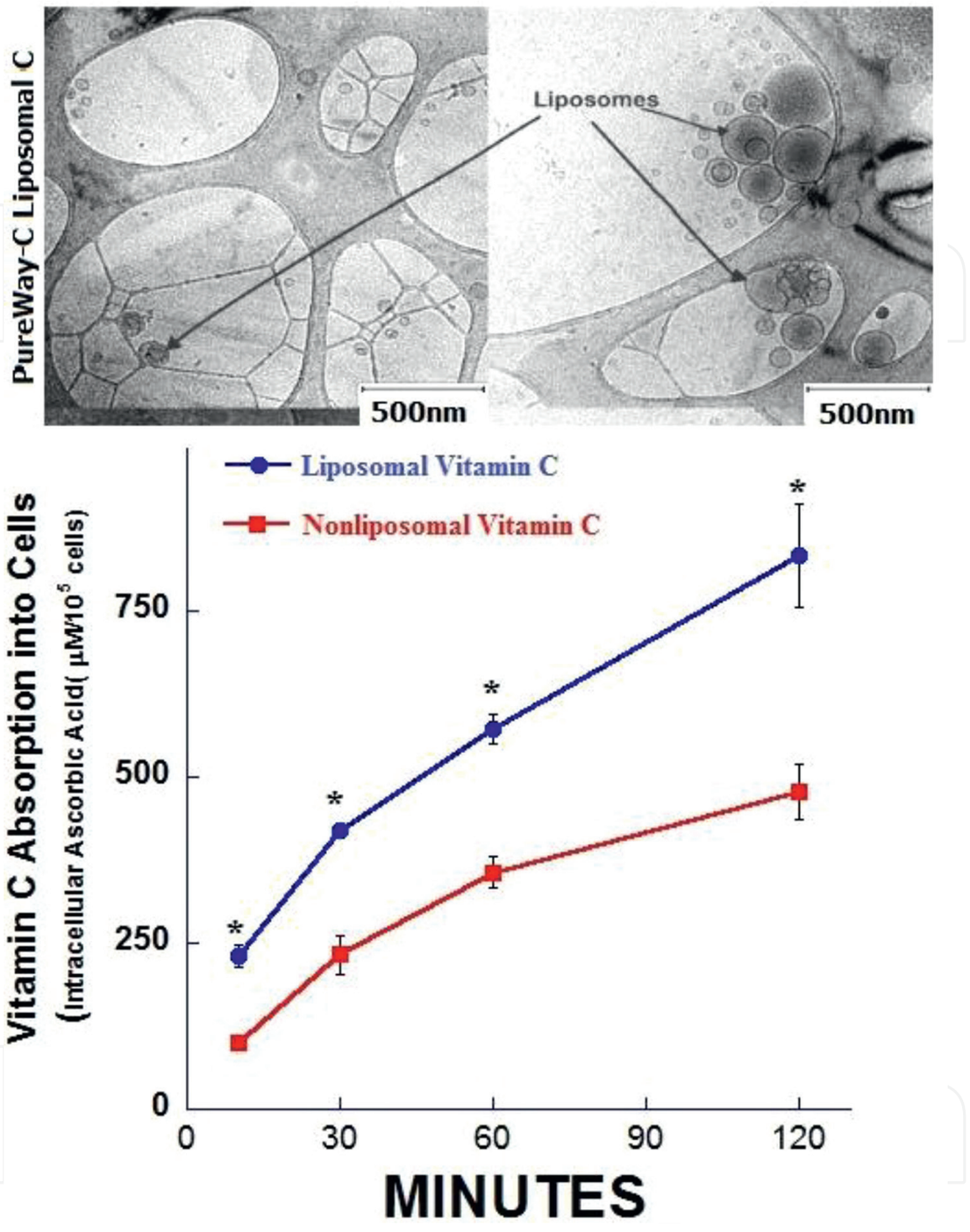

Figure 2.
PureWay-C® liposomal C is found in relatively small liposomes and better absorbed into human 786-0 epithelial cells. Top panel: TEM of PureWay-C® liposomal vitamin C (provided by one innovation labs, Miami, FL.) showing liposomes in the 100-250 nm range (top panel). The graph compares PureWay-C® liposomal C and nonliposomal vitamin C absorption into human epithelial cells. Human epithelial cells were seeded at 10^5 cells/well in 0.5 ml of serum free DMEM in wells of a 24 well tissue cluster plate. The cells were starved for 24 hours and then treated with 5 mM of liposomal vitamin C (blue line with circles) or nonliposomal vitamin C (red line with squares). The cells were incubated with the ascorbic acid for 10, 30, 60 and 120 minutes in triplicate after which the unabsorbed ascorbic acid was removed by aspiration and rinsing PBS, pH 7.2. The PBS was removed and the cells were lysed by three cycles of freeze-thawing for 10 minutes per cycle. The ascorbic acid EnzyChrom™ was then run on the cells lysate in the wells of the 24 well plate. When the chromagen was added the reaction was transferred to a 96 well plate and the OD was measured at 570 nm and the concentration of ascorbic acid was determined by comparison to a standard curve. The absorbed vitamin C is express as □M/10^5 cells. An ANOVA analysis of the data showed that the PureWay-C liposomal C demonstrated statistically significant better absorption that the nonliposomal C at all time points tested at 95% confidence (*).

3. Conclusions

Vitamin C is important for a wide range of biological activity and contributes to human health as an antioxidant, a co-factor for collagen synthesis and an immunomodulatory factor and also a wide range of additional biological activities that support skin, cardiovascular and nervous system health including anti-cancer properties [1–4]. Importantly, the effects of vitamin C on collagen synthesis and inflammation both contribute to wound healing and contributes to our understanding of Scurvy, as disease of failed wound healing due to the lack of dietary vitamin C [1–4]. Due to the importance of vitamin C in nearly all tissues, a great deal of research has gone into developing more bioavailable and better absorbed forms of vitamin C. While lipid-metabolite extraction had proved to provide the better absorbed vitamin C [79], recent research has found that packaging vitamin C into liposomes provides even better absorption than Ester-C® and PureWay-C® [82–85]. As noted, this improved absorption of liposomal vitamin C has been associated with improved immunological and cardiovascular function and protection from ischemia [82–85]. In addition to immune and cardiovascular function, research is also focused on the efficacy of vitamin C to enhance wound healing using the in vitro scratch assay. Indeed vitamin C does enhance wound healing in the in Vitro scratch assay [62–64]; (**Figure 1**). Further, liposomal vitamin C shows improved absorption into cells [82–85]; (**Figure 2**) which explains the improved bioactivity. Further advances in liposomal delivery vitamin C may be accomplished by incorporating recognition molecules into the lipid layer that can help with delivery. For example, proteins that bind to epithelial cells markers may even further increase liposomal vitamin C absorption into epithelial cells. Screening the bioactivity of these liposomal forms of vitamin C in the in vitro scratch assay is an excellent bioassay to continue to assess the efficacy of liposomal forms of vitamin C as they are developed.

Acknowledgements

Support for this research was provided by One Innovation Labs, 35 San Lorenzo Ave, Suite 850, Coral Gables, FL 33146 in the form of a grant "The role of Cannabidiol-TRKA interactions in neurite growth, T-cell attachment and epithelial cell migration." and supplies to Benjamin S. Weeks at Adelphi University, Garden City, NY 11530. Benjamin S. Weeks, Ph.D. has received support from One Innovation laboratories. One Innovation Laboratory also provided all fees associated with the publication of this Chapter.

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