Ascorbic Acid Reduces Oxidative Stress and the Percent Scratch Wound Healing in EA.hy926 Endothelial Cells

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Introduction

Ascorbic acid (AA), commonly known as Vitamin C, is an essential nutrient as it cannot be synthesized in the human body. It has a variety of biochemical functions including its roles in hydroxylation reactions for collagen synthesis and protecting cells against free radicals that are produced during metabolism (Chaitrakoonthong et al. 2020). Deficiency in ascorbic acid leads to scurvy, a disease characterized by bleeding gums, hemorrhage and ecchymoses caused by insufficient collagen production resulting in defective blood vessels (Telang et al. 2007). These symptoms reflect the crucial role of ascorbic acid in angiogenesis.

Angiogenesis is a prerequisite for tumor formation as it requires a vascular source to grow (Telang et al. 2007). Nitric oxide (NO) is a known stimulant of blood vessel formation. When oxidative stress disrupts the balance of NO, creating free radicals, the endothelium becomes damaged, leading to endothelial dysfunction and tumor angiogenesis (Rajendran et al. 2013). There have been several studies that have examined the antioxidative and anticancer effects of ascorbic acid, however results are contradictory. Antioxidants exhibit various effects on reactive oxygen species (ROS) production. They have been reported to produce free radicals, which may damage cells, and, in contrast, inhibit ROS production thereby protecting cells (Oh et al. 2020). Some studies demonstrate that ascorbic acid could quench ROS production by reducing the free radicals to a non-reactive state thereby stabilizing the mitochondrial membrane (Fukumura et al. 2012). These considerations provide general support for the idea that ascorbic acid supplementation might limit tumor growth by impairing blood vessel formation.

Based on data showing the antioxidant effects of ascorbic acid, we hypothesize that treating EA.hy926 endothelial cells with ascorbic acid will decrease the level of reactive oxygen species induced by hydrogen peroxide oxidative stress in comparison to the control condition. We will also analyze whether ascorbic acid will decrease the amount of cell migration and proliferation of EA.hy926 endothelial cells.

Methods and materials

Reagents and Kits

Hydrogen peroxide (H_2O_2), Dulbecco's Minimal Essential Medium, Fetal Bovine Serum (FBS), sodium hypoxanthine, aminopterin, and thymidine (HAT) media supplement and L-ascorbic acid were supplied by Sigma-Aldrich Chemical Co. The DCFDA / H2DCFDA - Cellular ROS assay kit was purchased from Abcam. All other reagents and chemicals were of chemical analytical grade.

Cell Culture

EA.hy926 cells were obtained from ATCC. The cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 25 mM D-glucose, and HAT media supplement. All cells were grown to confluence at 37 ℃ in humidified air containing 5% CO_2 (May and Qu 2011).

Measurement of Reactive Oxygen Species

Oxidative stress was analyzed by measuring the intracellular reactive oxygen species (ROS) levels using the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFDA / H2DCFDA - Cellular ROS assay kit) according to the manufacturer's instructions. Cells were plated in 24-well culture plates at a density of $1.0x10^5$ cells/well and allowed to attach for 24 hours. To induce oxidative stress, the cells were treated with 0.5mM of H_2O_2 (Seto et al. 2017). To assess the effects of ascorbic acid on EA.hy926 cells, the cells were incubated for 1 hour with 200 μM ascorbic acid followed by H_2O_2 (0.5mM). After incubation, cells were detached with Trypsin EDTA solution and washed with 100µL/well of 1×assay buffer. Cells were stained in culture media with 20 μM of the DCFDA dye and allowed to incubate for 30 min at 37 ℃. Oxidative stress was measured by analyzing the percent of DCFDA positive cells using flow cytometry at an excitation wavelength of 488 nm and emission wavelength of 535 nm (Abcam).

Scratch Wound Assay

To evaluate the wound healing effects of ascorbic acid on EA.hy926 cells, a scratch wound assay was performed. $1.0x10^6$ cells were seeded in a 35 mm dish with 2 ml of DMEM/10% FBS to create a confluent monolayer. After 24 hours, once cells reached confluence, a linear wound was made by scratching the cell monolayer with a 1 mm wide sterile plastic scraper (Tamilarasan et

al. 2006). The cells were then washed with PBS to remove any debris, treated with ascorbic acid (200 μM) and incubated for 4 and 8 hours at 37 ℃. After incubation, at each time point, dishes were placed under a phase-contrast microscope and were aligned in order to acquire an image (Liang et al. 2007). Images acquired for each sample were analyzed using ImageJ. The cell free area was measured in μm^2.

Statistical Analysis

Each trial experiment was repeated three times for each condition. All data are presented as the mean ± standard deviation and were analyzed using JASP software. Data analysis involved paired, two-way t-tests and the Bonferroni adjustment was used to correct for multiple comparisons. A value of $p<0.05$ for the DCDFA flow cytometry assay and a $p<0.0125$ for the scratch wound assay were considered to indicate a statistically significant difference.

Results

Antioxidative Effect of Ascorbic Acid by DCFDA Flow Cytometry

The results from the flow cytometry analysis demonstrated that under induced oxidative stress conditions, the addition of ascorbic acid decreased the percent of DCFDA positive cells and was significant for a p value of > 0.05 (Figure 1). The mean ± standard deviation for the two conditions were 79.1 ± 2.9 % and 55.4 ± 1.9 %, respectively.

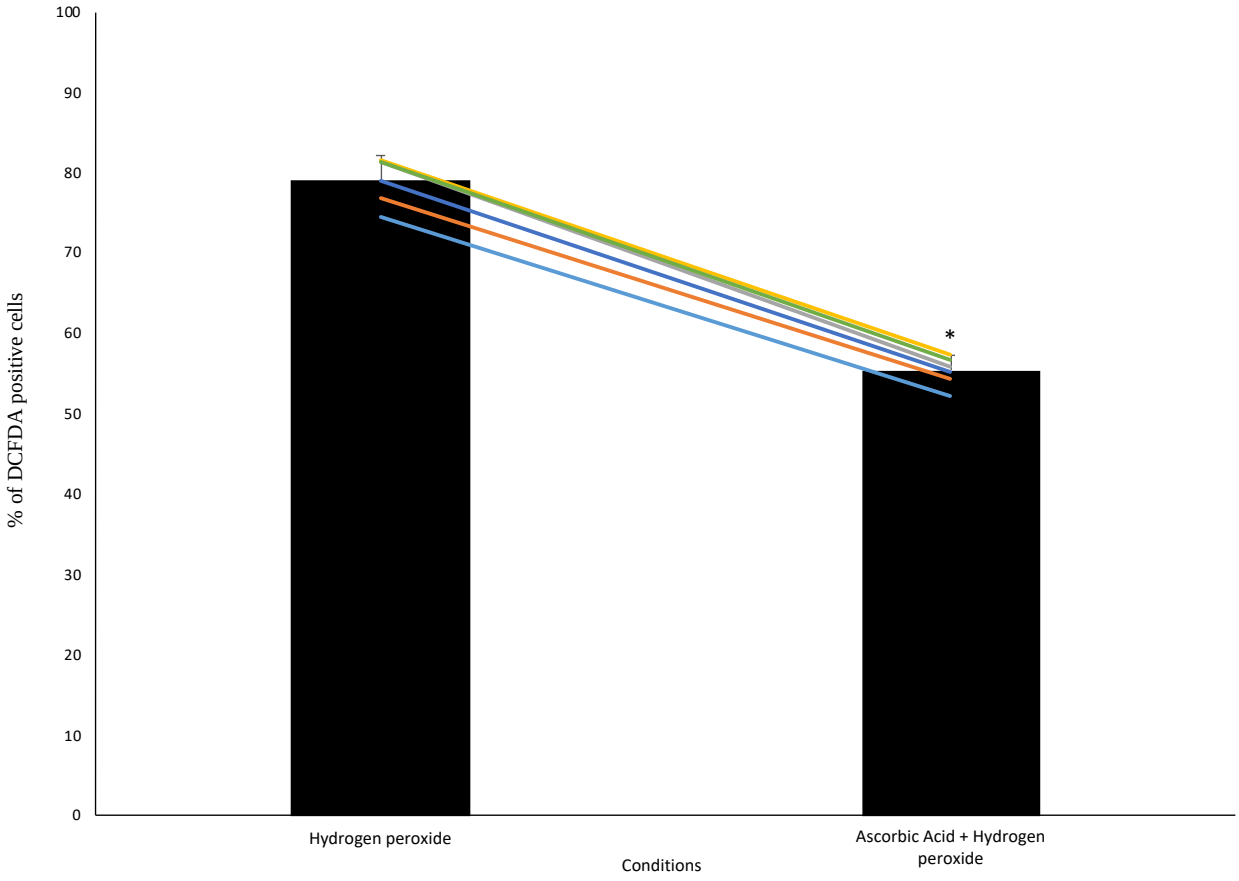
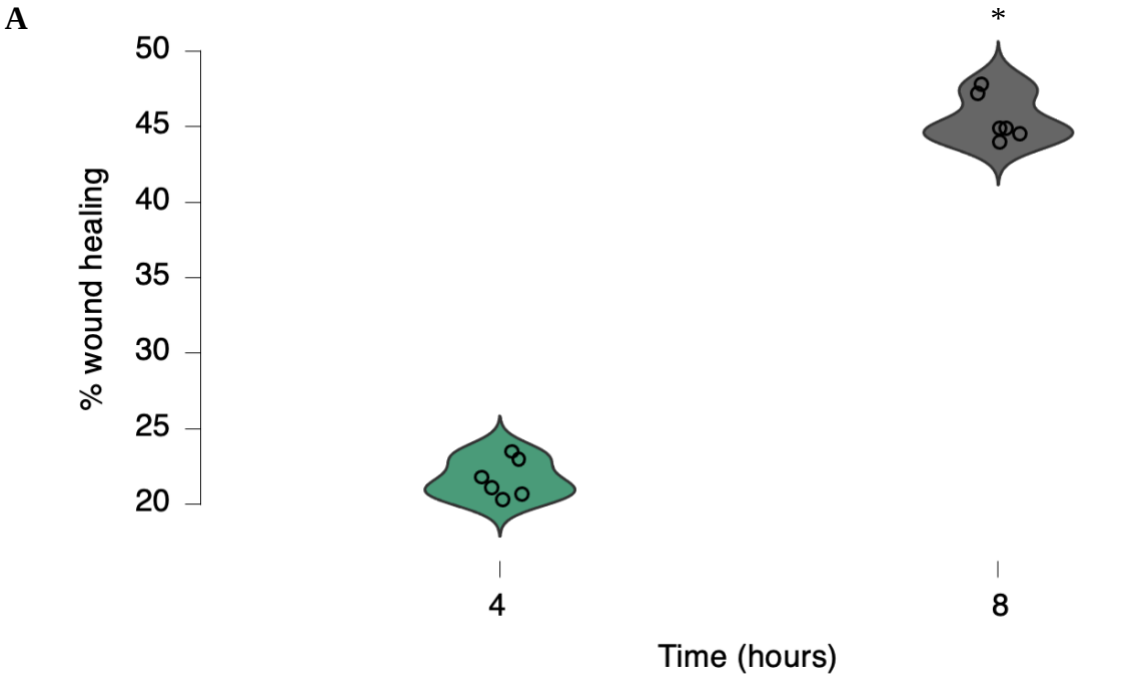


Figure 1. DCFDA assay with flow cytometry analysis of normalized hydrogen peroxide condition and ascorbic acid conditions. *significant for $p<0.05$

Scratch Wound Healing

The results of the scratch wound assay indicate that ascorbic acid significantly reduced wound healing compared to the control condition at both 4- and 8-hour time points ($p<0.0125$). In addition, significant wound healing occurred in both the control and ascorbic acid groups between 4 and 8 hours ($p<0.0125$). On average, the healing under control conditions at 4- and 8-hours was 21.7 ± 1.3% and 45.6 ± 1.5%, whilst under ascorbic acid conditions it was 8.15 ± 1.1% and 19.4 ± 1.2%, respectively (Figure 2).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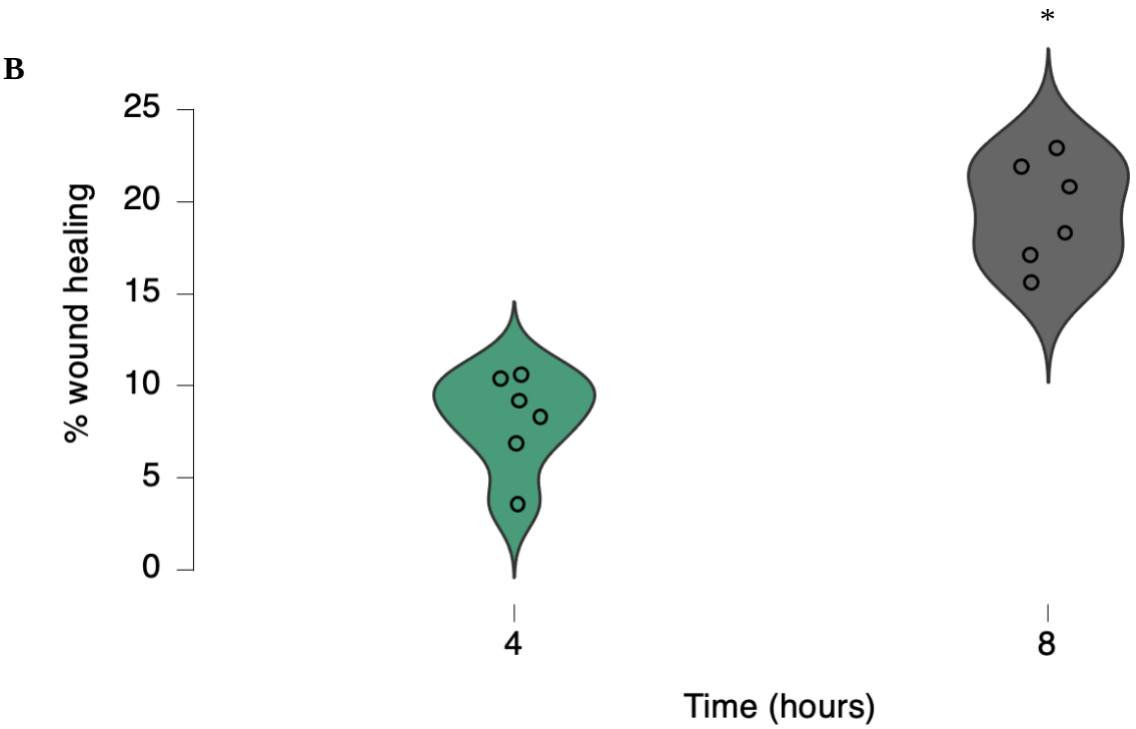
% wound healing
4
8
Time (hours)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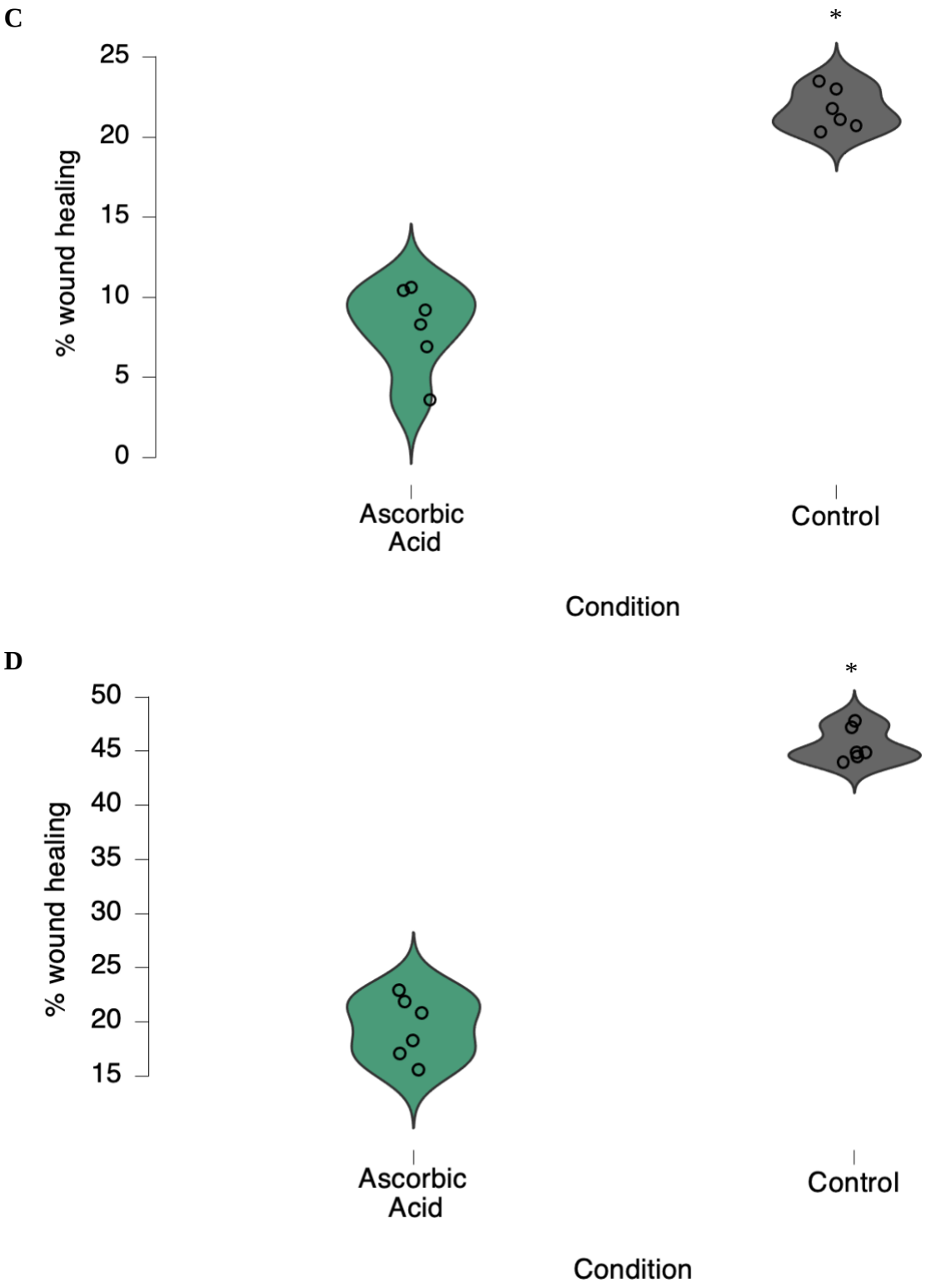

Figure 2. Scratch wound assay. (a) Percent wound healing of control group at 4 and 8 hours. (b) Percent wound healing of ascorbic acid group at 4 and 8 hours. (c) Percent wound healing between control group and ascorbic acid group at 4 hours. (d) Percent wound healing between control group and ascorbic acid group 8 hours. ∗significant for $p \leq 0.0125$

Discussion

In this study, the antioxidant properties of ascorbic acid on cell proliferation and migration were evaluated using a H_2O_2 induced oxidative stress model and scratch wound assay on EA.hy926 endothelial cells. Our experiment demonstrated that ascorbic acid reduced the amount of ROS generated by H_2O_2 as well as decreased the percent wound healing of the endothelial cells.

Ascorbic acid is a powerful antioxidant that scavenges reactive oxygen species, such as hydroxyl radicals, to prevent damage of vascular endothelium (Chaitrakoonthong et al. 2020). It consists of ionizable hydroxyl groups and, at physiologic pH, the ascorbate monoanion is the dominant form. It functions as a redox buffer, neutralizing reactive oxygen species, thereby preventing other compounds from becoming oxidized (Padayatty et al. 2014). In addition, it has been shown by other researchers that ascorbate prevents oxidation of tetrahydrobiopterin, a cofactor in nitric oxide synthesis (May et al. 2003). The vascular endothelium is important for the integrity of blood vessel as well in modulating vascular resistance. A decrease in nitric oxide is a characteristic of endothelial dysfunction caused by oxidative stress and is thought to play a major role in the development of angiogenesis in cancer (May et al. 2003). Our results are in accordance with a study performed by May and Qu (2011) in which the antioxidant function of ascorbic acid was analyzed, and a decrease in ROS production was found during oxidative stress induced by menadione. This is in contrast to other reports which found that ascorbic acid displayed pro-oxidant properties when transition metal ions were present (Kaźmierczak-Barańska et al. 2020). High pharmacological doses of vitamin C may induce pro-oxidant effects. These differing results suggest that further study is needed to determine the extent to which differing concentrations of ascorbic acid can protect endothelial cells from ROS.

The scratch wound assay assessed the impact of ascorbic acid on cell proliferation and migration. When we added vitamin C to cell cultures, we observed a significant delay in wound closure compared to the control group at both 4- and 8- hours. This indicates that ascorbic acid inhibited cell proliferation and migration and could suppress tumor angiogenesis. A potential explanation is that ascorbic acid and glucose have similar chemical structures. One mechanism by which ascorbic acid enters the cell is via the uptake of its two-electron oxidized form, dehydroascorbic acid, by glucose transporters (Kaźmierczak-Barańska et al. 2020). Therefore, an excess of ascorbic acid may compete with glucose for entry into the cell, thereby limiting the

amount of glucose available for ATP production resulting in less cell proliferation. This is in accordance with Yimcharoen et al. (2019) who demonstrated that, after exercising to induce oxidative stress, ascorbic acid supplementation could have interfered with glucose transportation and contributed to a lower production of ROS. Other studies have also shown that high doses of ascorbic acid reduced the amount of endothelial cell migration (Mikirova et al. 2008) while lower doses resulted in greater cell migration and tube formation (Telang et al. 2007). There are conflicting results when it comes to the effects of ascorbic acid on angiogenesis during tumor development. Some studies have reported suppression of tumor angiogenesis (in rat models) when treated with ascorbic acid while others showed a reduction of tumors in animals when depleted of ascorbic acid (Mikirova et al. 2008). As ascorbic acid plays an important role in the post-translational modifications (folding) of Type IV collagen required for angiogenesis (Telang et al. 2007), it would make sense that the addition of Vitamin C would aid in wound healing. However, our results indicate that the addition of ascorbic acid decreased cell proliferation and migration thereby inhibiting wound healing. This gives credence to the importance of Vitamin C in tumor angiogenesis and its potential anticancer properties.

In accordance with findings from other studies, high concentrations of ascorbic acid must be reached in order to be inhibitory (Mikirova et al. 2008). Concentrations high enough to inhibit tumor angiogenesis may not be feasible in humans via oral consumption (Mastrangelo et al. 2018). Although we demonstrated that ascorbic acid may decrease angiogenesis in vitro, this may not be attainable in vivo. In addition, it has been reported that free radicals produced by H_2O_2 are not efficiently scavenged by ascorbic acid (May et al. 2003). Therefore, it is possible that the ascorbic acid was inefficient in reducing the free radicals present. Future studies might focus on using different reactive oxygen species, such as xanthine oxidase, to induce oxidative stress and to evaluate the effects of ascorbic acid. In addition, the investigation of varying the ascorbic acid concentrations in order to optimize the scavenging of ROS and inhibition of tumor angiogenesis might be considered. In the long run, the effects of ascorbic acid on specific cancer cell angiogenesis might be explored.

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Appendix

Student's T-test at 95% confidence for flow cytometry analysis of DCFDA positive cells.

t-Test: Paired Two Sample for Means

	H_2O_2	$AA + H_2O_2$
Mean	79.08349201	55.43598995
Variance	8.276843706	3.577787183
Observations	6	6
Pearson Correlatior	0.958528131	
Hypothesized Mear	0	
df	5	
t Stat	48.56686998	
P(T<=t) one-tail	3.49625E-08	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	6.9925E-08	
t Critical two-tail	2.570581836	

Student's T-test with Bonferroni correction for % wound healing of control at 4 and 8 hours.

t-Test: Paired Two Sample for Means

	Control 4hrs	*Control 8hrs*
Mean	21.70807457	45.55676383
Variance	1.671396564	2.468627872
Observations	6	6
Pearson Correlation	0.674821448	
Hypothesized Mean Difference	0	
df	5	
t Stat	-49.3973142	
P(T<=t) one-tail	3.21257E-08	
t Critical one-tail	3.16338145	
P(T<=t) two-tail	6.42513E-08	
t Critical two-tail	3.8100047	

Student's T-test with Bonferroni correction for % wound healing of AA at 4 and 8 hours.

t-Test: Paired Two Sample for Means

	AA 4hrs	*AA 8hrs*
Mean	8.157661998	19.41095177
Variance	6.973081496	8.293318568
Observations	6	6
Pearson Correlation	-0.113021982	
Hypothesized Mean Difference	0	
df	5	
t Stat	-6.688327984	
P(T<=t) one-tail	0.000564825	
t Critical one-tail	3.16338145	
P(T<=t) two-tail	0.001129649	
t Critical two-tail	3.8100047	

Student's T-test with Bonferroni correction for % wound healing between control and AA at 4 hours.

t-Test: Paired Two Sample for Means

	Control 4hrs	*AA 4hrs*
Mean	21.70807457	8.157661998
Variance	1.671396564	6.973081496
Observations	6	6
Pearson Correlation	0.144682382	
Hypothesized Mean Difference	0	
df	5	
t Stat	11.99526384	
P(T<=t) one-tail	3.55155E-05	
t Critical one-tail	3.16338145	
P(T<=t) two-tail	7.1031E-05	
t Critical two-tail	3.8100047	

Student's T-test with Bonferroni correction for % wound healing between control and AA at 8 hours.

t-Test: Paired Two Sample for Means

	Control 8hrs	*AA 8hrs*
Mean	45.55676383	19.41095177
Variance	2.468627872	8.293318568
Observations	6	6
Pearson Correlation	-0.563541107	
Hypothesized Mean Difference	0	
df	5	
t Stat	16.08063345	
P(T<=t) one-tail	8.47091E-06	
t Critical one-tail	3.16338145	
P(T<=t) two-tail	1.69418E-05	
t Critical two-tail	3.8100047	

Descriptive Statistics for control at 4 and 8 hours.

	% wound healing	
	4 hours	**8 hours**
Valid	6	6
Missing	0	0
Mean	21.733	45.550
Std. Error of Mean	0.525	0.636
Std. Deviation	1.285	1.558
Variance	1.651	2.427
Minimum	20.300	44.000
Maximum	23.500	47.800

Descriptive Statistics for AA at 4 and 8 hours.

	% wound healing	
	4 hours	**8 hours**
Valid	6	6
Missing	0	0
Mean	8.167	19.433
Std. Error of Mean	1.072	1.175
Std. Deviation	2.625	2.877
Variance	6.891	8.279
Minimum	3.600	15.600
Maximum	10.600	22.900

Descriptive Statistics for AA and control at 4 hours.

	%	
	AA	**Control**
Valid	6	6
Missing	0	0
Mean	8.167	21.733
Std. Error of Mean	1.072	0.525
Std. Deviation	2.625	1.285
Variance	6.891	1.651
Minimum	3.600	20.300
Maximum	10.600	23.500

Descriptive Statistics for AA and control at 8 hours.

	%	
	AA	**Control**
Valid	6	6
Missing	0	0
Mean	19.433	45.550
Std. Error of Mean	1.175	0.636
Std. Deviation	2.877	1.558
Variance	8.279	2.427
Minimum	15.600	44.000
Maximum	22.900	47.800