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Collagen as a Nanonization Strategy for Enhancing Natural Antioxidants in Cancer Treatment

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ABSTRACT

The purpose of this research was to determine whether nanoscale resveratrol (RSV), collagen added to RSV, is more effective than regular RSV. Collagen is a sturdy emulsifier made in the human body (Cherng et al., 2013). RSV is an antioxidant which has anti-inflammatory properties (Cherng et al., 2013), allowing it to reduce reactive oxygen species (ROS) levels, which can induce cancer when in excess (Arnarson, 2019). However, RSV is not absorbed easily by the human body since it is excreted easily, making it less effective (Cherng et al., 2013). This research paper was focused on overcoming the low bioavailability of RSV through reducing particle size. The concept behind this is nanotechnology, using technology to study 1 to 100 nanometer structures (Bayda et al., 2019). Smaller RSV particles have increased surface area, which dissolves faster in the bloodstream (Deng et al., 2012). The hypothesis is that RSV particles would decrease by 8 times the volume, while the ROS levels in 3T3L1 cells would decrease with nanoscale RSV by 50%. The particle size distribution of RSV was measured with a spectrophotometer, while the ROS levels in 3T3L1 cells were measured by ELISA. The collagen-RSV solution had a greater peak than the regular RSV solution, indicating the particle size of RSV decreased after adding collagen. Also, the nanoscale RSV could reduce ROS levels more than regular RSV. This evidence supports the hypothesis that nanoscale RSV is effective in reducing ROS levels and is smaller than regular RSV.

Introduction

Cancer and Reactive Oxygen Species (ROS)

Cancer is one of the leading causes of death worldwide. In 2018, 9.5 million people died due to cancer (National Cancer Institute, n.d.). Seemingly beneficial as a cell signaling molecule, reactive oxygen species, or ROS, can induce cancer when in excess (Arnarson, 2019). ROS molecules are unstable particles formed from oxygen that are used by human immune cells to fight infection (Arnarson, 2019). However, if ROS particles exceed the amount of antioxidants, ROS particles accumulate and induce widespread apoptosis, which leads to DNA damage and tumorigenesis (Arnarson, 2019). RSV, a natural polyphenol that is sourced from plants, induces cell apoptosis in tumors. RSV does so in many ways, such as activating the function of p53, which is a tumor suppressor gene, modifying cyclin-dependent kinase levels to cause cell cycle inhibition, and increasing Fas ligand levels in signaling pathways (Ko et al., 2017). RSV is a type of antioxidant (Cherng et al., 2013). Antioxidants can neutralize excess ROS by giving their electrons to the unpaired electrons in the ROS molecules (Arnarson, 2019). Because of its ability to decrease ROS levels and its natural origin that improves health, RSV is a crucial natural chemical to study. If the bioavailability of RSV were to be improved in its effectiveness, it could become a potential treatment option for cancer pairing with other drugs, as well as an anti-aging agent in the beauty industry.

Nanoscience and Collagen in Chemical Delivery

Collagen is a flexible and sturdy structure that makes up 25% to 35% of the human body's protein (Deshmukh et al., 2016). It is produced by connective tissue cells such as fibroblasts (Deshmukh et al., 2016), as well as being mostly found in connective tissue like cartilage and skin (Wang, 2021). Being the main component of the extracellular matrix (ECM), collagen forms fibrous strands that act as a scaffold for supporting fragile organs (Wang, 2021). Its notable properties of biodegradability, biocompatibility, and simplicity of being processed make collagen a versatile material to work with (Cherng et al., 2013). As a result, collagen has multiple uses in different fields, such as the food industry and the medical field (Hashim et al., 2015).

Nanoscience is the study of structure or molecules ranging from lengths of 1 nanometer(nm) to 100 nm (Cherng et al. 2013). In 1959, American physicist Richard Feynman first introduced the nanotechnology concept in a 1959 lecture called "There's Plenty of Room at the Bottom" (Khan et al., 2019). In 1974, nanotechnology, the usage of nanoscience in technology such as devices, was coined and first used by Japanese physicist Norio Taniguchi (Bayda et al., 2019). In a scientific paper describing the concepts of nanotechnology, he defined nanotechnology as separating, consolidating, or deforming an atom or molecule (Taniguchi, 1974). After 20 years of research and breakthroughs, nanotechnology has become one of the most useful and promising fields of the 21st century. Techniques of nanotechnology include the top-down approach, which is separating a block into smaller particles, and the bottom-up approach, which creates nanoscale creations through atoms (Khan et al., 2019). Technology needed for these methods include precision engineering, which creates nanostructures using sensors to control size and number; lithography, and the atomic force microscope and the scanning probe microscope (Bayda et al., 2019).

RSV anticancer activity against several types of cancers

Resveratrol (3,5,4′-trihydroxy-trans-stilbene, RSV) is a natural polyphenol, antioxidant, and an emulsifier that is found in several foods such as grapes, peanuts, and red wines (Cherng et al., 2013). RSV has antioxidant and anti-inflammatory properties, as well as high membrane permeability which allows it to pass the blood-brain barrier and stomach epithelium barrier (Cherng et al., 2013). Proven by past studies, RSV has been shown to diminish cell proliferation and prevent strictures in Crohn's disease patients (Garcia et al., 2012). It has also been used to increase apoptosis in fibroblasts via downregulating mRNA transcription of procollagen type I and II, which could be applied to patients with hypertrophic scar disorder (Zeng et al., 2013). In addition, RSV is able to target multiple types of cancer cells, including breast, skin, colon, and ovary cancer cells (Ko et al., 2017). RSV inhibits cancer cells by suppressing free radical formation, such as those produced by 12-*O*-tetradecanoylphorbol-13-acetate in human leukemia HL-60 cells (Sharma et al., 1994). RSV inhibits cancer cells by inhibiting expressions of certain enzymes, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), in human breast cells (Chen et al., 2004). RSV downregulates expressions of cyclin D2 and cyclin E in skin cancer cells to inhibit their development, and upregulates p21 expression, a crucial gene for inhibiting cell growth (Gartel et al., 2022). Most importantly, RSV induces apoptosis in many pathways, such as the PI3K/Akt/mTOR pathway in multiple cancer cells (Bai et al., 2010).

Limitation of RSV's efficacy for cancer treatment

However, RSV has its drawbacks in pharmacokinetic abilities, such as that it has low bioavailability. When RSV is turned into glucuronic acid and sulfate conjugations, its double bond is hydrogenated, along with other structural changes when RSV is degraded (Neves et al., 2012). These changes make RSV less permeable to cells, causing RSV to be excreted more easily (Neves et al., 2012). In addition, it has low water solubility, leading to the weak binding ability of plasma proteins, so RSV will not be distributed throughout the body (Neves et al., 2012). Therefore, the majority of RSV is metabolized and excreted by the liver and the concentration of RSV will be at a low level in the

bloodstream (Cherng et al. 2013). This makes RSV an inefficient drug because larger doses of RSV must be taken to achieve the same therapeutic level of other water-soluble drugs. This research hopes to overcome the low bioavailability of RSV through reducing the particle size of RSV. Smaller RSV particles have increased surface area, which dissolves faster in the bloodstream (Sun et al., 2012). This way, RSV can become a cost-effective treatment option.

The question proposed for this research was "How does collagen affect the particle size of RSV and level of reactive oxygen species inside 3T3L1 cells?" The hypothesis was that the particles would decrease by 8 times the volume, while the ROS levels in the 3T3L1 cells with nanoscale RSV would decrease by 50%. For results, collagen decreased RSV particle size by a maximum of 8 million times, and decreased ROS levels by approximately 21%. To take away, these results show that collagen could be an effective emulsifier that is able to lower RSV particle size significantly, and that RSV is useful for decreasing ROS levels in 3T3L1 cells.

Methods

Study Design

The independent variable in this research was the collagen-RSV mixture, which is the nanoscale RSV. This variable was chosen to see the effects of collagen as an emulsifier at different concentrations when applied to RSV. To accurately manipulate the independent variable, the different amounts of collagen were planned before conducting the experiment, and conversions were made to form the correct concentrations. In addition, the experiments were done in a biosafety cabinet, while a lab coat and gloves were worn. The dependent variables were the particle size distribution of RSV and the ROS levels measured in the 3T3L1 cells. These variables were beneficial to study because they directly showed the effects of collagen on reducing RSV's particle size, as well as compared the amount of ROS levels reduced in nanoscale RSV compared to regular RSV.

There were two parts of the experimentation: measuring the RSV's particle size and the ROS levels in the 3T3L1 cells. Solutions of collagen mixed with RSV and another solution of regular RSV were made, then measured with a spectrophotometer to see the particle size distribution of each solution. This would reveal which solution had smaller particle sizes and therefore measure the effectiveness of collagen reducing RSV particle size. For measuring the ROS levels, 3T3L1 cells were grown and incubated in an incubator. 3T3L1 cells were chosen because their fiber-like appearance easily reveals how they are affected by different chemicals. Then, different concentrations of collagen-RSV and RSV were prepared in test tubes beforehand, then applied to the 3T3L1 cells.

Materials Used

The following materials were used throughout the experiments: RSV (MedChemExpress, Cat#: HY-16561), collagen (Sigma-Aldrich, Cat#:C5483), ethanol (Sigma-Aldrich, Cat#:Ex0290, EtOH) and acetic acid (Sigma-Aldrich, A6283, AcOH). The ethanol was used to dissolve RSV, and the AcOH was used to dissolve collagen. The concentrations of RSV, collagen, ethanol, and AcOH are detailed in the experimental procedures below. The 96-well plate of 3T3L1 cells were seeded according to the manufacturer's instructions and incubated for 24 hours at 37°C.

Measurements of Particle Size Distribution of RSV

First, micropipette tips and RSV, collagen, ethanol, and AcOH were sterilized for 30 minutes at 121°C, then dried overnight. Second, 900 µL of ethanol and 100 µL of AcOH were each added to two 1.5 mL Eppendorf tubes. Third, 1mg of RSV was weighed by the electronic scale and added to ethanol and AcOH from the last step. Fourth, 2.5 µL of collagen was added to 900 µL of ethanol and 100 µL of AcOH in an Eppendorf tube. The two solutions created were sent to The Department of Biomedical Engineering at the National Taiwan University to measure its particle size

distribution using a spectrophotometer (Flinn Scientific, Cat#: AP7596). The spectrophotometer measures the particle size distribution in nanometers, and their relative quantities in percentages.

Measurement of ROS Levels in 3T3L1 Cells

For measuring the ROS levels in 3T3L1 cells, two experiments were conducted with different concentrations of collagen and RSV. The following materials are used: 1 ROS Detection Assay Kit (Biovision, Cat#ab287839) which includes an ROS inducer, a full 96-well plate containing equal amount of 3T3L1 cells in each well (Bioresearch Collection and Research Centre), RSV, collagen, ethanol, AcOH, and Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Cat#12491015). The DMEM was modified by adding 10% Fetal Bovine Serum (Thermo Fisher Scientific, Cat#10437036). The 96-well plate (Sigma-Aldrich, Cat#CLS3922) was first seeded with equal amounts of 3T3L1 cells in 36 of the wells, and 100 µL of DMEM was pipetted into those 36 wells. The 3T3L1 cells were incubated at 37°C overnight. Then, 9 mL of DMEM and 36 µL of ROS inducer were mixed well. In addition, 0.25% of collagen and 1000 µL of AcOH was added in the previous mixture. The concentrations of collagen and RSV in different groups are shown in the Appendix (Appendix Figure 1, Appendix Figure 2). Each concentration was repeated six times. The cell plate was inserted into an ELISA plate reader (Fisher Scientific) and measured for its fluorescence intensity in Relative Fluorescence Units (RFU).

Statistical Analysis

After the fluorescence intensity data is gathered, the averages of the fluorescence intensity in each group of cells were calculated and compared. In addition, a T-test was performed, as well as finding the standard deviation and the probability value (P-value). The T-tests were two-tailed distributions and the unequal variance type. Probability values were yielded from the T-tests. For the second experiment, all steps were repeated from the first experiment, with two exceptions: 96 wells of the 96-well plate were seeded and used, and 16 Eppendorfs were used, containing a wider range of concentrations of collagen and RSV. Bar graphs were created.

Results

Collagen reduces particle size distribution of RSV

When measuring the effects of collagen on the particle size distribution of RSV, a line graph was created from the particle size distribution of RSV compared to nanoscale RSV, which was created from a spectrophotometer (Figure 1). The line representing RSV is neon green, and the line representing nanoscale RSV is red. There are four major peaks throughout the graph. At 1 nm, nanoscale RSV has a 2 percent greater peak compared to RSV. At 50 nm, the nanoscale RSV has a 2 percent lower peak compared to RSV. At 500 nm, the nanoscale RSV has a 5 percent greater peak compared to RSV. At 7000 nm, The line representing RSV added to collagen had a greater peak at 1 nm than RSV only. Overall, the first peak decreases to the second peak, then increases in height at the third and fourth peak. When comparing the volume of the regular RSV to the reduced RSV, at 1 nm, there is a ratio of 8,000,000 to 1. To summarize, collagen decreases RSV's particle size at a maximum of 8 million times at lower concentrations, but at higher concentrations, collagen increases the particle size of RSV.

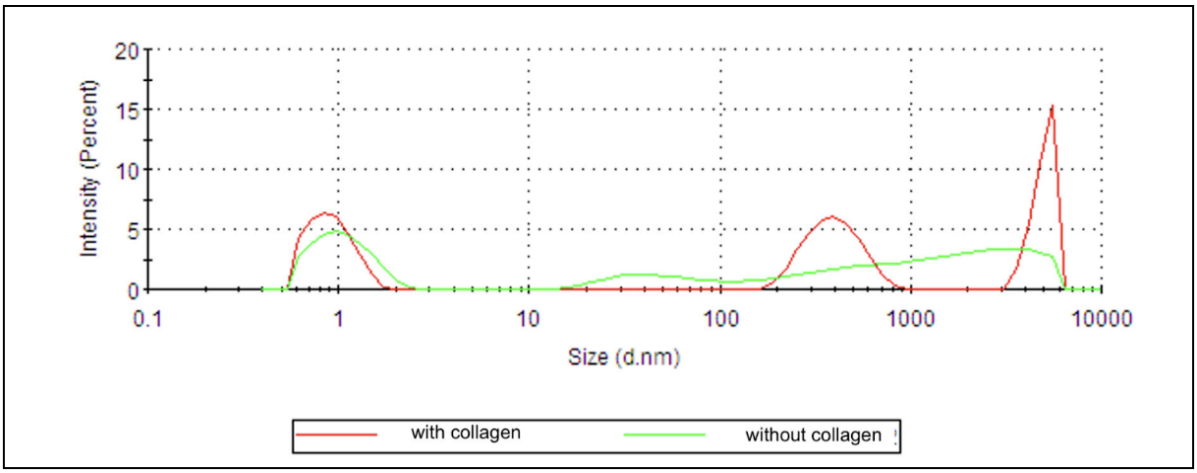

Figure 1. Nanoscale RSV's particle size is decreased at lower concentrations and increased at higher concentrations. Particle size distribution graph of RSV compared to nanoscale RSV (n=2). A solution of RSV and a solution of nanoscale RSV were inserted into a spectrophotometer.

ROS levels in 3T3L1 cells was reduced by nanoscale RSV Treatment

For measuring the ROS levels in 3T3L1 cells, the cell plates were inserted into the ELISA microplate reader to measure fluorescence intensity (Figure 2 and Figure 3). For Experiment 1 (Figure 2), all the averages of the fluorescence intensity values were around 0.9, besides the AcOH control + 10 nM RSV group, which had a 1.01 average. There is a statistically significant value of less than 0.01 when comparing the control to the Collagen 0.25% + 50 nM RSV group, as well as a statistically significant value of less than 0.001 when comparing the 30 min. ROS Inducer to the Collagen 0.25% + 50 nM RSV group, showing that the nanoscale RSV's decrease in ROS levels is significant. In the bar graph for Experiment 1, there is a gradual decrease in the ROS level from the control group to the Collagen 0.25% + 50 nM RSV group (Figure 2), showing that the nanoscale RSV's decrease on ROS levels is dose-dependent. In addition, the decrease in ROS levels, from the 30 min. ROS Inducer to the Collagen 0.25% + RSV 50 µM, is 21%.

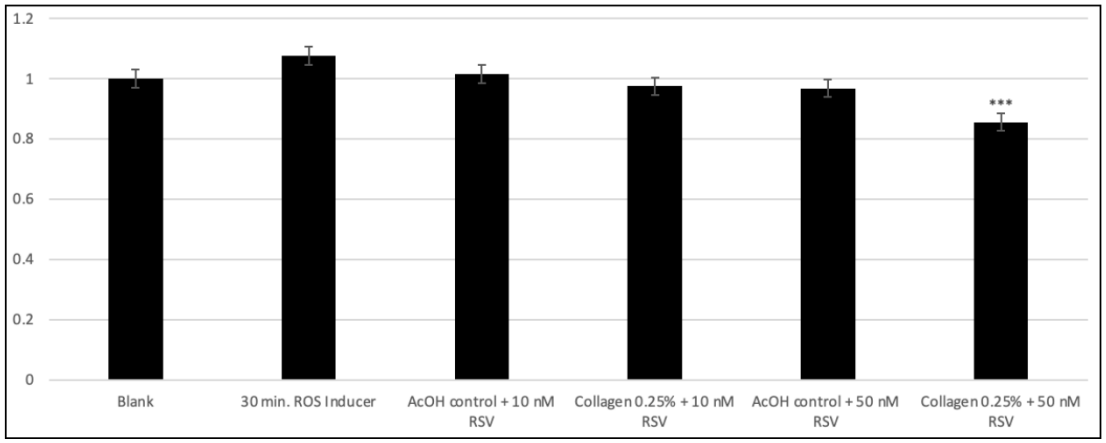

Figure 2. ROS levels in 3T3L1 cells in Experiment 1 were reduced after treatment of nanoscale RSV. Bar graph of ROS levels in 3T3L1 cells (n=1). A 96-well plate was seeded for 36 wells and different concentrations of chemicals were added to each group. All groups besides the control group were induced with ROS for 30 minutes. Then, it was inserted into the ELISA plate reader to measure fluorescence intensity. Excel was used. Statistical significance is indicated by significance stars, with one star being <0.05, two stars being <0.01, and three stars <0.001.

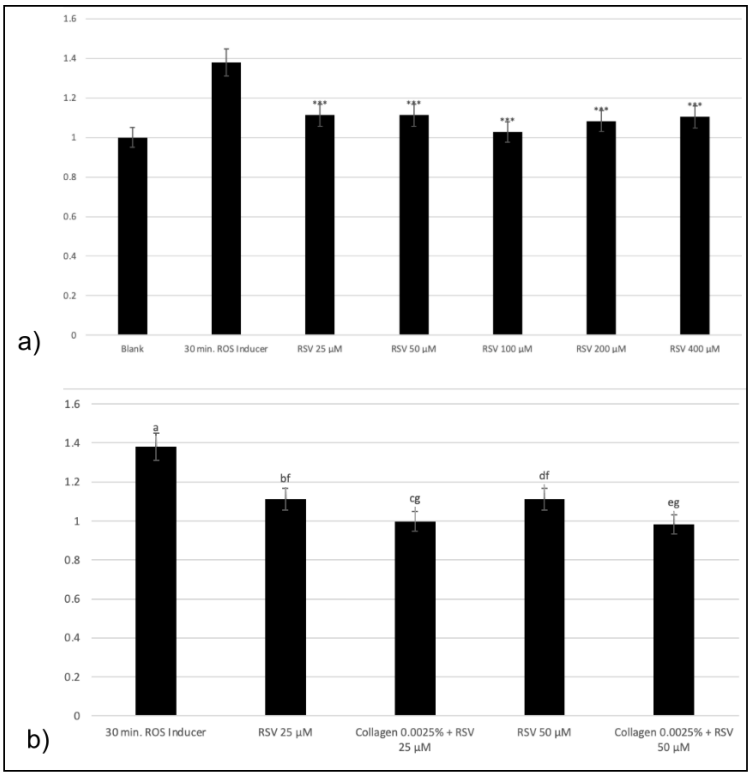

Figure 3. ROS levels in 3T3L1 cells in Experiment 2 were reduced after treatment of nanoscale RSV. Bar graph of ROS levels in 3T3L1 cells (n=1). Excel was used. a) ROS levels in 3T3L1 cells in different concentrations of RSV. Statistical significance is indicated by significance stars, with one star being <0.05, two stars being <0.01, and three stars <0.001. b) ROS levels in 3T3L1 cells with collagen added to different concentrations of RSV. Bars labeled with different letters share a statistical significance, while bars labeled with the same letters share no statistical significance.

For Experiment 2 (Figure 3), most of the averages of the fluorescence intensity values were around 1.1, besides the cell group with Collagen 0.0025% + RSV 25 µM and Collagen 0.0025% + RSV 50 µM, which had averages of around 0.9. There was a statistically significant value of less than 0.001 when comparing the 30 min. ROS Inducer group to the RSV 25 µM group, the RSV 50 µM group, the RSV 100 µM group, the RSV 200 µM group, and the RSV 400 µM group (Figure 3a), showing that RSV is able to decrease ROS levels in 3T3L1 cells. In addition, there was a statistically significant value of less than 0.01 when comparing the RSV 25 µM group to the Collagen 0.0025% + RSV 25 µM group, and a statistically significant value of less than 0.001 when comparing the RSV 250 µM group to the Collagen 0.0025% + RSV 50 µM group. This result showed that collagen is able to significantly decrease ROS levels compared to RSV alone. The bars in the bar graph decreased slightly if collagen is added, then increased slightly if there is no collagen added. In addition, the decrease in ROS levels, from the 30 min. ROS Inducer to Collagen 0.0025% + RSV 50 µM, was 40%.

Discussion

As a natural antioxidant, RSV is a supplement with anti-cancer properties. However, it has low bioavailability, making it hard to be absorbed by the small intestine. If RSV were improved in its effectiveness, it could potentially treat cancer patients, in a drug cocktail form, in the future. Due to these properties, RSV is an important supplement to study.

Whether hypothesis was supported

The hypothesis of this research paper is partially supported because after adding collagen to regular RSV, the RSV particles decreased by, in fact more than, eight times in volume. However, the ROS levels did not decrease by as much as 50%. For the particle size distribution graph of RSV and nanoscale RSV, the nanoscale RSV has a greater peak than the regular RSV at the 1 nm and the 50 nm mark. In addition, when comparing the volume of the regular RSV to the reduced RSV at 1 nm, the ratio is 8,000,000 to 1. This shows that collagen decreased the particle size of RSV by 8 million times, creating more smaller-sized particles within the solution. For the ROS levels of 3T3L1 cells, there were two experiments conducted (Figure 2 and Figure 3). For Experiment 1, the decrease in ROS levels, from the 30 min. ROS Inducer to the Collagen 0.25% + RSV 50 μM, is 21%. For Experiment 2, the decrease in ROS levels, from the 30 min. ROS Inducer to Collagen 0.0025% + RSV 50 μM, which is 40%. These results indicate that 50 μM of RSV added to collagen is the most effective concentration in reducing ROS levels. The decrease of ROS levels are all less than 50% of the control group values, which does not match the hypothesis. However, for Experiment 2, there are statistically significant values less than 0.01 when comparing the 30 min. ROS Inducer group to all the groups with collagen added to RSV. The statistical significance shows that nanoscale RSV is more effective than regular RSV in reducing ROS levels.

Properties of Nanoscale RSV

As an anti-aging supplement, RSV has many beneficial properties such as anti-inflammatory and high membrane permeability (Cherng et al., 2013), allowing it to be applied towards treating many diseases. First, RSV is able to decrease proliferation of fibroblasts by decreasing mRNA expression of type I and III procollagen, causing it to be a potential treatment for hypertrophic scar (Zeng et al., 2013). In addition, RSV causes cell-cycle arrest in intestinal smooth muscle cells, meaning it could potentially treat Crohn's disease (Garcia et al., 2012). Thirdly, due to its anti-inflammatory and anti-thrombosis properties, RSV could treat COVID-19 symptoms (Giordo et al., 2021). RSV also induces cell apoptosis in cancer tumors, allowing it to be a treatment option for cancer when paired with other drugs in a drug cocktail (Koushki et al., 2018). Because nanoscale RSV is derived naturally from plants, RSV will be a healthier, less invasive treatment option for future cancer patients. Nanoscale RSV retains all the properties of RSV and helps RSV overcome its low membrane permeability. Nanoscale RSV is more easily absorbed by the small intestine, which makes the supplement more cost effective and more likely to be used widely by hospitals during treatment. Besides treating diseases using RSV, this study also showed the effectiveness of collagen as an emulsifier. Compared to other methods of nanotechnology, collagen is relatively simple and does not need other machinery to work. Collagen can be used in several fields such as the food industry as natural preservatives or taken as supplements to strengthen tissues which become weaker as humans age (Hashim et al., 2015).

Conclusion

The results of this project were not completely expected, as it was thought RSV alone would have a greater effect on the ROS levels in 3T3L1 cells, due to its antioxidant abilities. However, nanoscale RSV having both larger-sized particles and smaller-sized particles was expected, since the solutions of nanoscale RSV and RSV were hand-mixed. To improve my future research, the particle size, molecular weight, and charge of collagen should be specified, since these parameters could have an effect on emulsification. In addition, electronic mixers should be used instead of hand-stirring. This study about collagen's effect on RSV particle size and ROS levels can be connected to other studies in this field. This study showed that collagen reduced the particle size of RSV so that it became nanoscale, which aligns with the data that nanoscale RSV is reduced to 100-150 nm in diameter in production of nanoscale RSV particles (Cherng et al., 2013).

After the research conducted in this paper, a question that appeared was what are the effects of nanoscale RSV on live subjects compared to regular RSV? To extend this research, a new collagen-RSV solution could be made, improved by data collected from the particle size distribution graph as well as the ROS levels in the 3T3L1 cells. In addition, all aspects of the collagen will be specified, and non-toxic substances will be used to dissolve collagen and RSV. The solution would then be applied to live vertebrate animals for further research.

In hindsight, this research shed light on its potential role in collagen, which affects the particle size of RSV by acting as an emulsifier and reduced the particle size 8 million times. In addition, collagen reduces the ROS levels in 3T3L1 cells by at most 40%. Therefore, collagen could be an effective emulsifier in RSV nanonization, boosting RSV's ability to treat multiple diseases. Due to its potential, nanoscale RSV should continue to be studied.

Limitations

Limitations to the study are displayed in the particle size distribution graph as well as the bar graphs for ROS levels in 3T3L1 cells (Figure 1, Figure 2, and Figure 3). In the particle size distribution graph, the nanoscale RSV has a greater peak at the 500 nm and the 7000 nm mark compared to the regular RSV. This means that collagen was unable to reduce the size of the larger-sized particles, causing regular RSV to have less larger-sized particles. This result could have been caused by the uneven mixing of the RSV with collagen, since the solutions were hand-mixed and not with an electronic stirrer. Therefore, collagen could have not emulsified RSV completely.

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