Kale Remediation of Stressed 3T3 Cells

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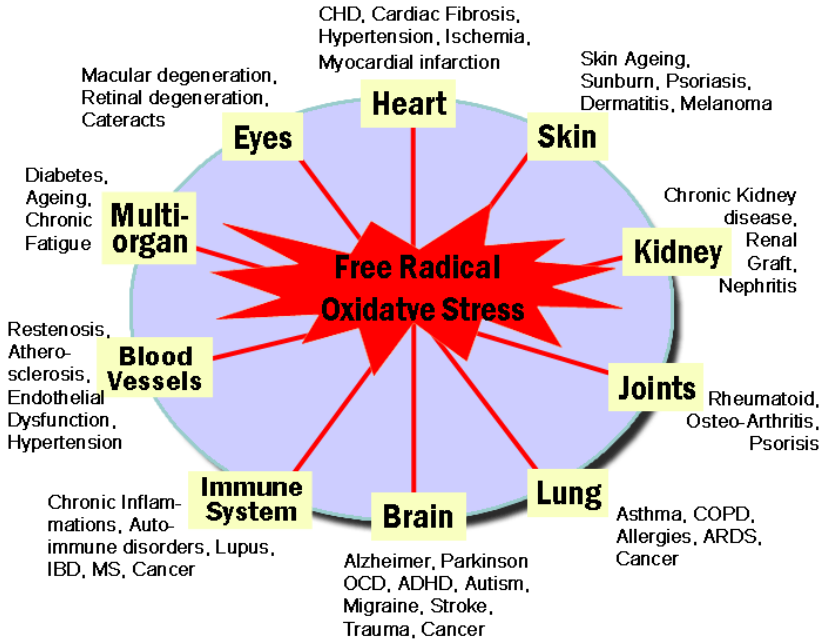
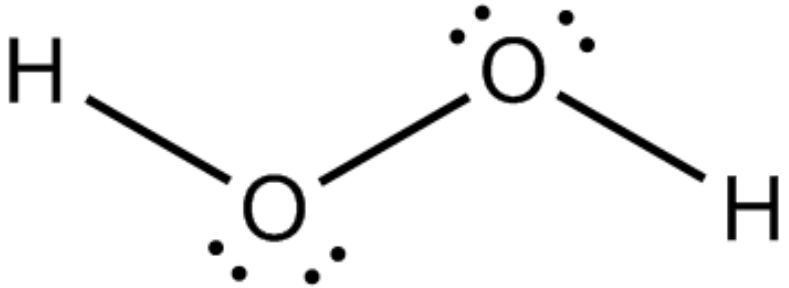
Stress

- Anything that disrupts regular cellular processes and reduces survivorship
- In sudden emergencies, cells create stress proteins that are programmed to repair damage that the stress induces
- Stress proteins often restore damaged proteins to normal shape, thus restoring function
  - Programmed to cope against infection and autoimmune disease

Oxidative Stress

- Increases oxidant production in cells
- Free radicals of the reactive oxygen species (ROS) can damage cells
- Reactive species may cause direct cell death or induce cancer
- **Hydrogen Peroxide** (H_2O_2) used to cause oxidative stress



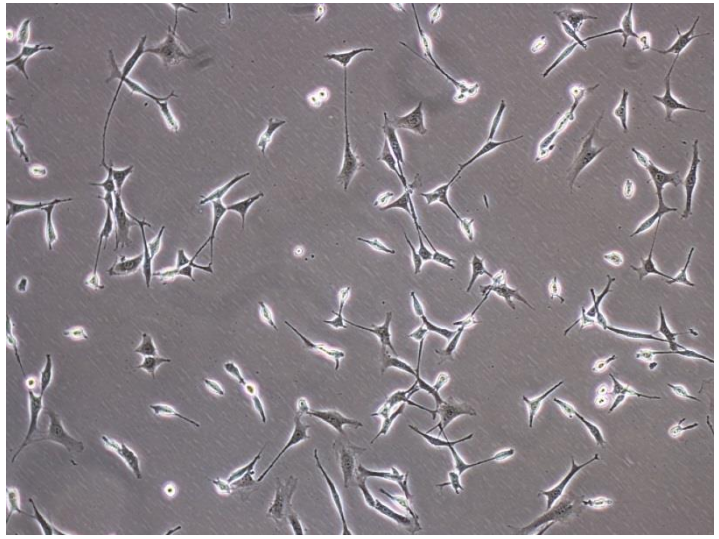


Kale (*Brassica oleracea*)

- Vegetable widely known for its health benefits
  - Very high in beta-carotene, vitamin K, vitamin C, and calcium
- A source of indole-3-carbinol
  - Boosts DNA repair in cells
- Additional source of two carotenoids, lutein and zeaxanthin

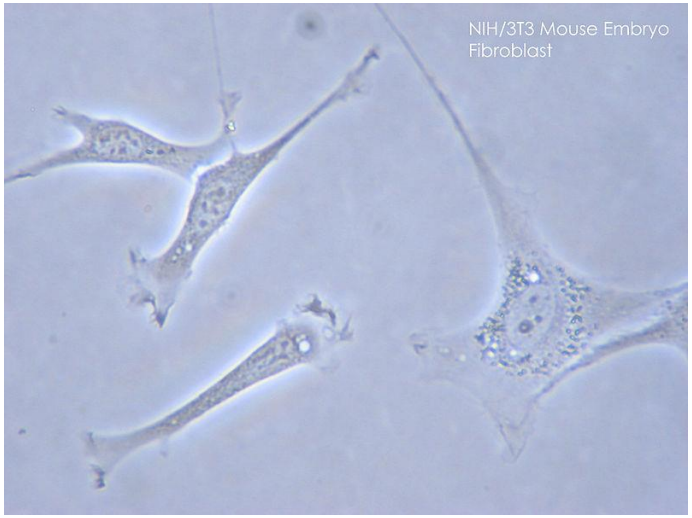
Mammalian Cell Lines

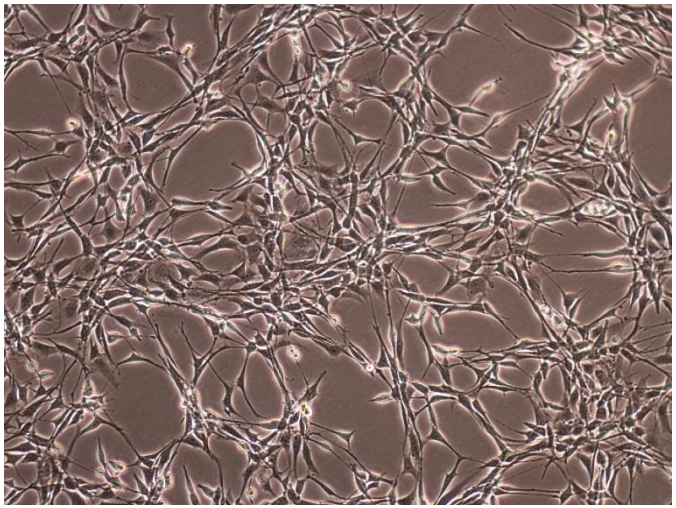
- Can be induced to self-renew indefinitely and generate all cell types of the organ from which they originate
- Mainly studied in humans and model organisms, such as mice and rats



3T3 Cell Line

- Cell line established from Swiss mouse embryo tissue
- Has become the standard fibroblastic cell line
- Often used in the cultivation of keratinocytes (skin cells), with the 3T3 cells secreting growth factors favorable to these kinds of cells





Purpose

- To determine the effects of kale extract and oxidative stress on the proliferation of 3T3 cells

Hypotheses

Null Hypotheses

- Kale and hydrogen peroxide, individually, **WILL NOT** significantly affect the rate of 3T3 cell proliferation
- Kale **WILL NOT** significantly remediate the oxidative stress of hydrogen peroxide on 3T3 cells

Alternative Hypotheses

- Kale and hydrogen peroxide, individually, **WILL** significantly affect the rate of 3T3 cell proliferation
- Kale **WILL** significantly remediate the oxidative stress of hydrogen peroxide on 3T3 cells

Materials

- Cryotank
- Two 75mm^2 tissue culture treated flasks
- Twenty-four 25 mm^2 tissue culture treated flasks
- Fetal bovine serum (FBS)
- Trypsin-EDTA
- Pen/strep
- Macropipette + sterile macropipette tips (1 mL, 5 mL, 10, mL, 20 mL)
- Micropipettes + sterile tips
- DMEM Serum - Complete Media (4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate + 10% fetal bovine serum)
- 3T3 Fibroblastic Cell Line
- Kale extract
- Hydrogen Peroxide
- 75 mL culture flask
- Incubator
- Nikon Inverted Microscope
- Laminar Flow Hood
- Laminar Flow Hood UV
- Sterilizing Lamp
- Labeling Tape
- Hemocytometer
- Sterile PBS
- Ethanol (70%)
- Purple Nitrile gloves

Procedure: Cell Culturing

- 1 mL aliquot of 3T3 cells from a Cryotank was used to inoculate 30 mL of DMEM media in two 75mm^2 culture flask, yielding a cell density of approximately 10^6 to $2x10^6$ cells/mL.
- Media was replaced with 15 mL of fresh media to remove cryo-freezing fluid and incubated (37° C, 5% CO_2) for 2 days until a cell density of approximately $4x10^6$ to $5x10^6$ cells/mL was reached.

Procedure: Day 0 (Addition of Variable)

- After trypsinization, cells in all flasks were pooled into 1 common 75mm^2 flask (cell density of approximately 1 million cells/mL).
- 1 mL of cell suspension was added to twenty-four 25 mm^2 tissue culture treated flasks containing 3.5 mL of DMEM media, creating a cell density of approximately 10^5 cells per flask.
- The stock solution of kale extract (100X) was created by steaming and blending 10 grams of kale in water (combined volume of stock = 100 mL), followed by cheese cloth, filtering, and sterile filtering.
- The following concentrations of variable(s) (next page) were added to the flasks. 24 total flasks for experiment(12 for stress or non-stress, 6 for each day, and 2 for each concentration of variable).
- Cells were incubated at 37°C, 5% CO_2 for the remainder of the study.

Concentrations of Variable

Non-Stress Experiment:

	0	X	10X
Stock	0 mL	5 µL	50 µL
Media	5 mL	4.995 mL	4.950 mL
Total	5 mL	5 mL	5 mL

Stress Experiment:

	0	X	10X
Stock	0 mL	5 µL	50 µL
H_2O_2	10 µL	10 µL	10 µL
Media	4.990 mL	4.985 mL	4.940 mL
Total	5 mL	5 mL	5 mL

- **X equals the estimated concentration of the variable present in the fluid compartments of the body**

Procedure: Days 1 and 3 (Counting/Imaging)

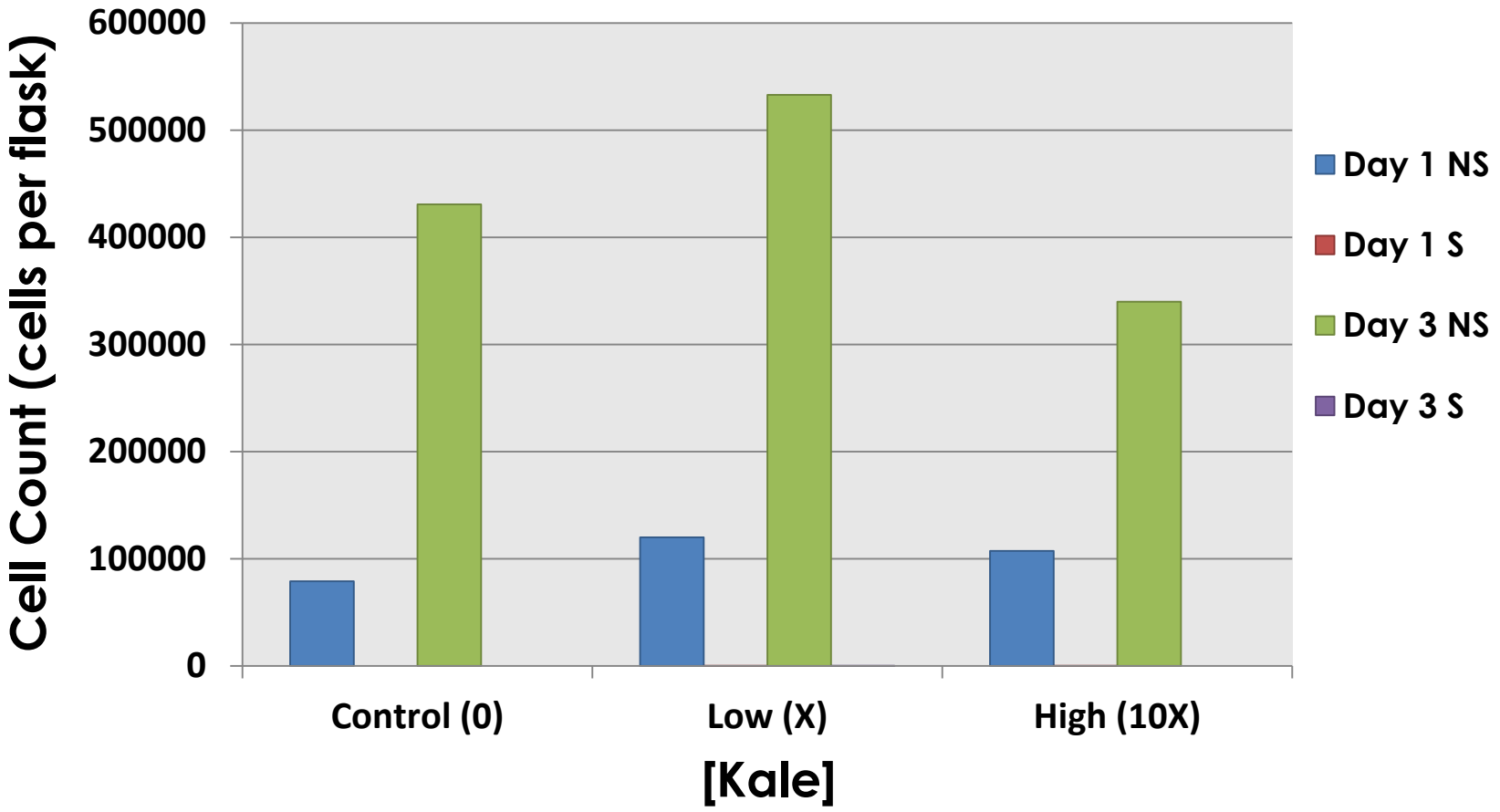
- Day 1
  - Using the flasks from each group, cell densities were determined as follows:
    - The cells were trypsinized and collected into cell suspension.
    - 25 µl aliquots were transferred to a Hemocytometer for quantification (eight counts per flask).
- Day 1 and Day 3
  - The previous procedure for determining densities was used again, and a Nikon Inverted Microscope was used to take images of representative areas of each flask.

Statistical Analyses of the Proliferation Results

- ANOVA (Single/Double Factor)
  - Compares variation within groups to variation between groups
  - Using the ANOVA, a P-value less than the alpha of **.05** was gathered (significant variation)
  - Reject the null hypothesis
- Dunnett's Test
  - Compares each experimental group to control individually
  - 0.05 alpha was used, and the T-value compared to the T-critical value of **2.42**

Results of 3T3 Proliferation Analysis

Day #, NS/S	Day 1 NS	Day 1 S	Day 3 NS	Day 3 S
P-value	8.656×10^{-19}	0.360	1.784×10^{-18}	0.376



Dunnett's Test Results

$$t_d = \frac{M_i - M_c}{\sqrt{\frac{2MSE}{n_h}}}$$

Concentration	T-Value	T-Critical (0.05)	Variation
Day 1, NS	-	-	-
X	15.159	2.42	**Significant**
10X	10.445	2.42	**Significant**
Day 3, NS	-	-	-
X	8.058	2.42	**Significant**
10X	7.156	2.42	**Significant**

Double-Factor ANOVA Results

Day # (Variable Tested)	P-Value	Effect Significance
Day 1	-	-
Oxidative Stress	5.598×10^{-91}	**Significant**
Kale Concentration	3.314×10^{-26}	**Significant**
Combination	4.310×10^{-26}	**Significant**
Day 3	-	-
Oxidative Stress	2.967×10^{-87}	**Significant**
Kale Concentration	1.245×10^{-25}	**Significant**
Combination	1.300×10^{-25}	**Significant**

Conclusions

- Kale
  - As suggested by the Double ANOVA and Dunnett's Test statistical analyses, both concentrations of kale significantly affected 3T3 cell proliferation as seen on Days 1 and 3.
  - **(Reject 1st Null Hypothesis)**
- Oxidative Stress
  - As suggested by the Double ANOVA statistical analysis, the oxidative stress of hydrogen peroxide significantly affected 3T3 cell proliferation as seen on Days 1 and 3.
  - **(Reject 1st Null Hypothesis)**
- Synergistic
  - As suggested by the Double ANOVA statistical analysis, a combination of both variables significantly affected 3T3 cell proliferation as seen on Days 1 and 3. However, the kale concentrations did **NOT** remediate the oxidative stress.
  - **(Accept 2nd Null Hypothesis)**

Future Changes

Limitations

- Only 1 cell line was used
- Only 1 concentration of hydrogen peroxide was used
- Hemocytometer counts in proliferation subject to technique/clumping errors

Extensions

- Obtain an LD-50 for hydrogen peroxide
- Other quantitative proliferation tests (MTT assay, etc.)
- Use a wider range of concentrations
- Synergistic effects of kale with other substances?

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