L-ARGININE REMEDIATION OF STRESSED 3T3 FIBROBLAST CELLS

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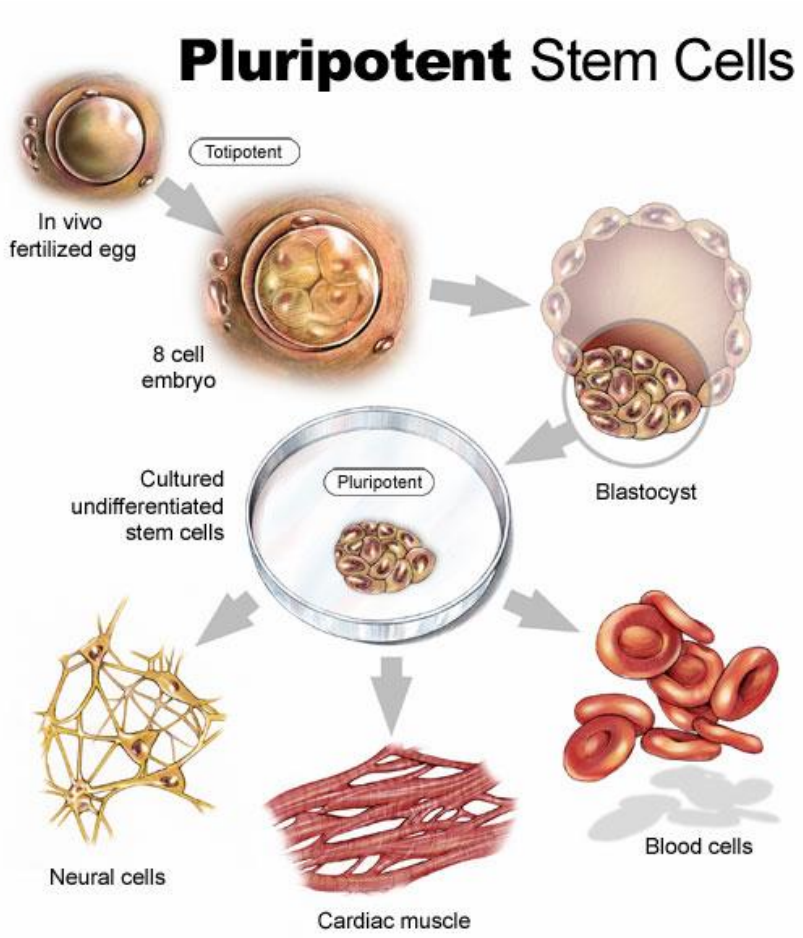
Tissue Engineering

- What is TE?
  - The development and manipulation of artificial implants, laboratory-grown tissues, and genetically engineered cells and/or molecules to replace or support the function of defective or injured body parts
- Why is TE important?
  - It has the potential to replace or supplement the function of tissues destroyed or compromised in any variety of ways, including:
    - Inherent design flaws
    - Hereditary/congenital defects or conditions
    - Disease
    - Trauma
    - Damage from an individual's environment
    - Aging
- TE has great potential for supplementing muscle tissue.

Adult Stem Cells

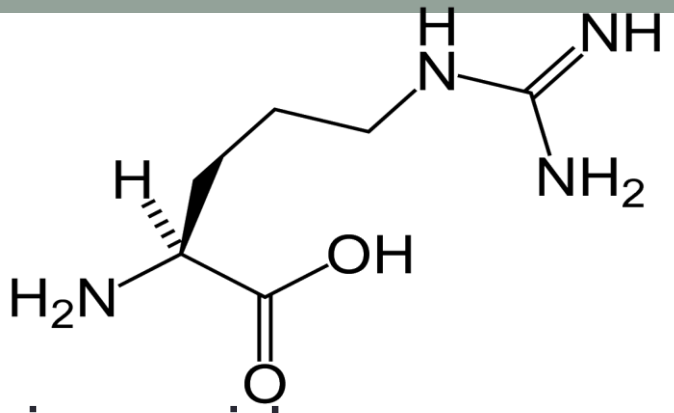
- Undifferentiated cells multiply to replenish dying cells
- Somatic stem cells, can be found in juvenile and adult animals and humans
- S*elf-renew* indefinitely, and generate all cell types of the organ from which they originate
- They have mainly been studied in humans and model organisms such as mice and rats



3T3

- Cell line established from Swiss mouse embryo tissue
- standard fibroblast cell line
- Do not differentiate, produce ECM parts and structural proteins
- Often used in the cultivation of keratinocytes, with the 3T3 cells secreting growth factors favorable to these kinds of cells.

L-arginine



- One of the 20 most common natural amino acids
- Semiessential amino acids for mammals
- Produced by the body
- Precursor for the synthesis of nitric oxide
- Reduces healing time of injuries (bone)

Oxidative stress

- H_2O_2 stress
- Increases oxidant production in cells
- Free Radical accumulation can lead to compounding stress
- Results in cellular degeneration
- May cause direct cell death or induce cancer
- High glucose levels induce an increase in enzymatic activity leading to the synthesis of amino acids

Purpose

To determine the remediation effects of L-arginine on the proliferation of stressed 3T3 fibroblast cells.

Hypotheses

- Null Hypothesis: L-arginine will not significantly aid the survival of H_2O_2 stressed 3T3 fibroblast cells

- Alternative Hypothesis: L-arginine will significantly increase survivorship of 3T3 fibroblast cell

Materials

- Cryotank
- 75mm^2 tissue culture treated flasks
- 25 mm^2 tissue culture treated flasks
- Fetal bovine serum (FBS)
- 3T3 Fibroblastic Cell Line
- Trypsin-EDTA
- Pen/strep
- Macropipette + sterile macropipette tips (1 mL, 5 mL, 10, mL, 20 mL)
- Micropipettes + sterile tips
- DMEM Serum - 1% and Complete Media (4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate + [10% fetal bovine serum for complete])

- 75 mL culture flask
- L-arginine
- H_2O_2
- Incubator
- Nikon Inverted Microscope with imaging technology
- Laminar Flow Hood
- Laminar Flow Hood UV Sterilizing Lamp
- Sharpie pen
- Hemacytometer
- Sterile PBS
- Ethanol (70%)
- Sterile Water
- Purple Nitrile gloves

Procedure (Cell Line Culture)

- A 1 mL aliquot of 3T3 cells from a Cryotank was used to inoculate 30 mL of 10% serum DMEM media in a 75mm^2 culture flask yielding a cell density of approximately 10^6 to $2x10^6$ cells
- The 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
- The culture was passed into 12 flasks in preparation for experiment and incubated for 2 days at 37° C, 5% CO_2

Procedure (Addition of Variable on Day 0)

- Cells from a T75 flask were resuspended after trypsinization to a density of approximately 300-500K/mL. T75 flasks were incubated for 4 minutes at 37° C
- 4 mL of 10% DMEM media was added to each T25 flask
- 0.5 mL of cell suspension was transferred to 12 T25 flasks. Flasks were placed back into incubator and cells were allowed to attach for several hours
- 1g of pure L-arginine powder was added to 10mL of 70% ethanol (C_2H_6O). This created a stock of 1000x, where x indicates the estimated concentration in the liquid compartments of the body.
- T25 flasks were removed from incubator and variable and H_2O_2 was added to reach desired concentrations

Concentrations

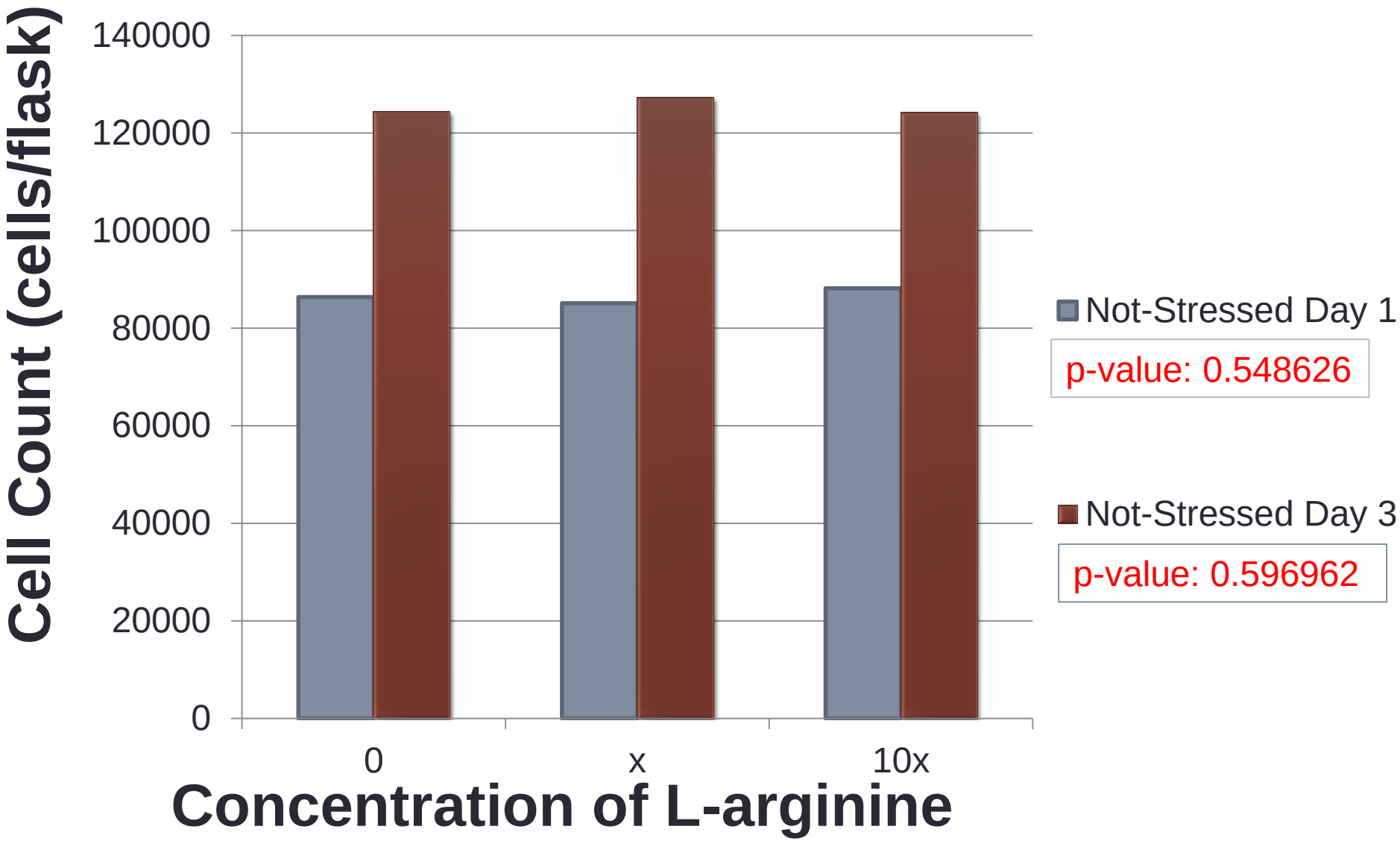
Stressed	0	X	10x
L-arginine	0 mL	5 µL	50 µL
H_2O_2 (3% solution)	15µL	15 µL	15 µL
Media	5 mL	4.980 mL	4.935 mL
Total	5 mL	5 mL	5 mL

Non-Stressed	0	X	10x
L-arginine	0 mL	5 µL	50 µL
Media	5 mL	4.995 mL	4.950 mL
Total	5 mL	5 mL	5 mL

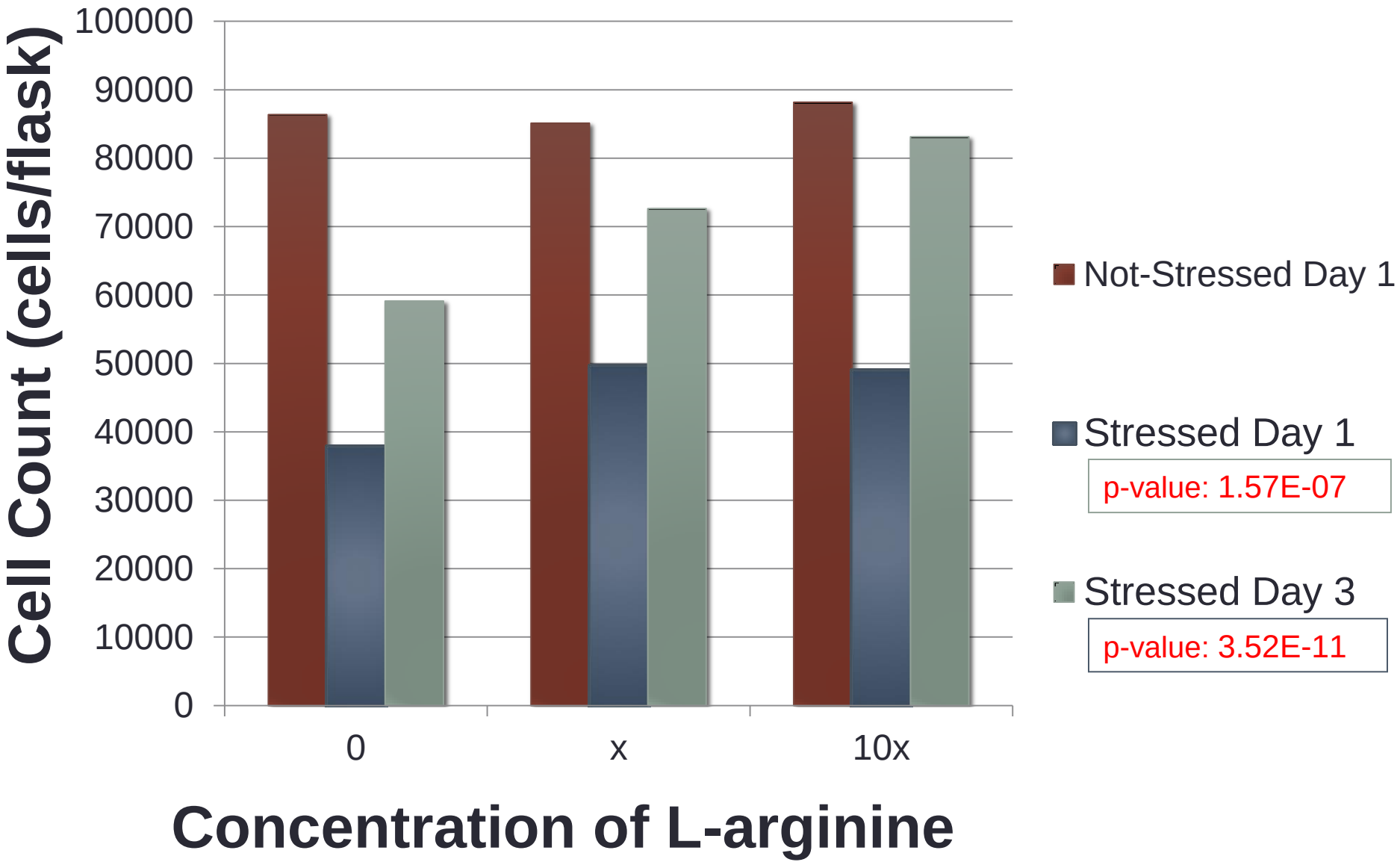
Procedure (Cell Counts)

- Day 1 and Day 3
  - 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 total counts).

Non-Stressed Results



Stressed Results



Dunnett's Test Results

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

Concentration	T-Value	T-Critical (0.05)	Variation
Stressed Day 1	-	-	-
X	5.98	2.67	**Significant**
10X	5.66	2.67	**Significant**
Stressed Day 3	-	-	-
X	5.32	2.67	**Significant**
10X	9.46	2.67	**Significant**

Statistical Analysis

Proliferation P-Values					
Non-stressed Day 1	Non-stressed Day 3	Stressed vs Non-stressed Day 1	Stressed vs Non-stressed Day 3	Stressed Day 1	Stressed Day 3
0.548626	0.596962	2.85E-17	7.65E-19	1.57E-07	3.52E-11
Not Significant	Not Significant	Significant	Significant	Significant	Significant

Conclusions

- Null hypothesis can be rejected.

Conclusion

- L-arginine does not significantly affect non-stressed 3T3 fibroblast cell proliferation
- H_2O_2 does significantly affect 3T3 fibroblast cell proliferation
- L-arginine does significantly affect the survival of H_2O_2 stressed 3T3 fibroblast cells

Project Limitations and Extensions

- **Limitations**
  - Hemacytometer counts can vary
  - Cell clumping
  - Low number of flasks
  - Lag time
- **Extensions**
  - Use a wider range of concentrations
  - Test the effects of L-arginine on other cell lines (C2C12, MG63)
  - Test synergistic effects
  - CyQUANT™ Cell Proliferation Assay
    - More quantitative than counting cells on a hemocytometer
    - Fluorescent dye binds to nucleic acid in cell

Acknowlegments/Works Cited

- Dr. Phil Campbell
- Conrad M. Zapanta, Ph.D. Biomedical Engineering Laboratory, Carnegie Mellon University
- Mark Krotec, PTEI
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