

Quantifying ROS Production in L-cells Exposed to Heat Stress Using DCFH-DA Fluorescence

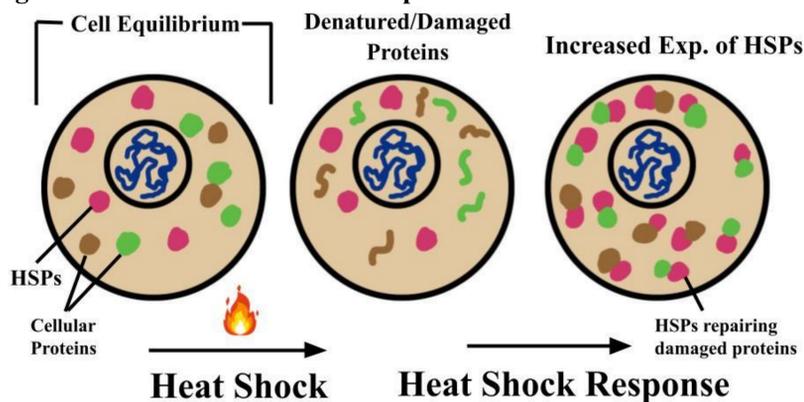
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Introduction

Reactive Oxidative Species

A reactive oxidative species (ROS) is a highly reactive oxygen containing molecule that quickly interacts with other molecules in the cell causing **oxidative stress**. ROS are produced during mitochondrial respiration in which one-electron transfer produces free radical oxygen species. Low level ROS are required for cell signaling pathways however, when the concentration of ROS is too great for the cell's antioxidants to control reactive oxidative species, chain reactions occur, **damaging DNA, RNA, and proteins**. ROS can be deadly if not properly regulated. This study examines the relationship between heat shock (Figure 1) and oxidative stress. The continued characterization of oxidative stress and its causal factors are instrumental in the understanding and treatment of many oxidative stress and aging related diseases including Alzheimer's and Parkinson's Disease.

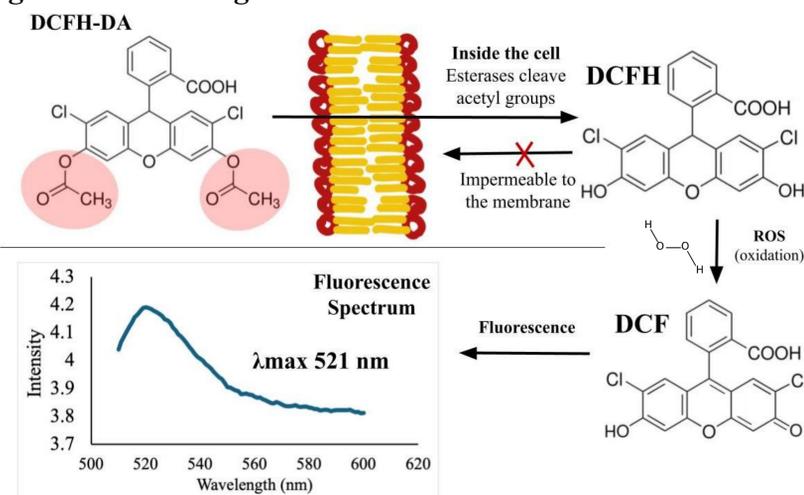
Figure 1. Cellular Heat Shock Response



Measuring Oxidative Stress

2, 7-Dichlorofluorescein diacetate (DCFH-DA) is commonly used as an oxidative stress quantification tool (Kim, 2020). A flow diagram showing how DCFH-DA is used is shown in Figure 2. When DCFH is oxidized to DCF by an ROS like hydrogen peroxide (H_2O_2), the molecule becomes fluorescent. The excitation wavelength for DCF is 500 nm and emission at 521 nm. The membrane permeability of DCFH-DA and impermeability of DCFH along with the fluorescence characteristics of DCF makes the chemical a suitable quantitative indicator of oxidative stress.

Figure 2. Flow Diagram of DCFH-DA to DCF in the Cell



Methods

Table 1. Experimental Conditions in DCFH-DA ROS Quantification

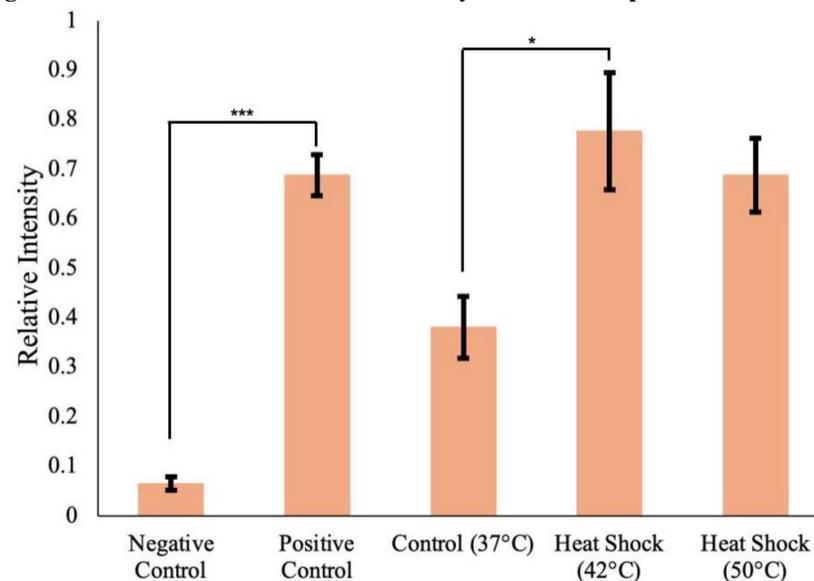
| Reagent or Variable | Conditions | | | | | |
|--|------------|------------------|------------------|--------------|------|------|
| | Blank | Negative Control | Positive Control | Exp. Control | 42°C | 50°C |
| ~200,000 Cells | Y | Y | Y | Y | Y | Y |
| Q.S. PBS | Y | Y | Y | Y | Y | Y |
| H ₂ O ₂ (peroxide) | N | Y | Y | N | N | N |
| DCFH-DA | N | N | Y | Y | Y | Y |
| Incubation Temp | 37°C | 37°C | 37°C | 37°C | 42°C | 50°C |

In determining whether heat shock causes an increase in oxidative stress in L Cells (CRL-2648), experimental conditions were prepared as shown in Table 1.

L Cells (CRL-2648) were cultured in a growth media solution of Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Calf Serum (FCS), and 1% Penicillin-Streptomycin (PS) at 37°C, and 5% CO₂. A 10 μ M DCFH-DA solution prepared immediately prior to the experiment in 1% DMSO and PBS was kept wrapped in aluminum foil away from light. It was crucial that the DCFH-DA solution was kept out of light exposure until its addition to the experimental solution as to avoid premature DCFH-DA degradation.

Results

Figure 3. DCFH-DA Fluorescence Intensity in L-Cells Exposed to Heat Stress



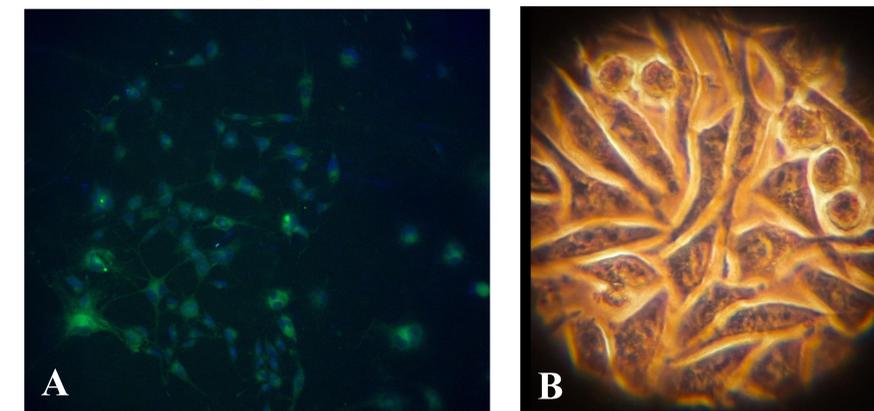
Heat stress conditions at 42°C and 50°C increased oxidative stress relative to the normal growth condition (37°C). T-tests revealed a statistical increase in DCF fluorescence intensity between the control condition (37°C) and the first heat shock condition, 42°C ($p = 0.041$). DCF fluorescence intensity did not change significantly from the first heat shock condition to the second (50°C) heat shock condition ($p = 0.56$). The increase in fluorescence intensity between the negative control and the positive control was statistically significant ($p = 0.00014$).

Relative DCF intensity was calculated by subtracting the background fluorescence signal from the total fluorescence using a standardized blank solution. (* = $p < 0.05$, *** = $p < 0.001$)

Fluorescence Microscopy

DCFH-DA and DAPI were used as fluorescent probes in fixed L-cells. DAPI selects for the nucleus and fluoresces blue light while DCF is present in the cytoplasm and fluoresces green light. Figure 4A shows the distribution of DCF in the cell and how it does not appear to be taken up into the nucleus. However, due to cells often emitting baseline green fluorescence, confidence in DCF location within the cell is low and the image is instead qualitative. Figure 4B contains an image of highly confluent L-cells exposed to white light.

Figure 4. A) Fluorescence Imaging of L-Cells with DAPI and DCF, B) Light Microscopy of Highly Confluent L-Cells



Discussion

The cell's heat shock response **increases mitochondrial activity** to combat the negative effects of excessive heat exposure. When a cell is exposed to a heat shock, proteins and membranes denature and become metastable or nonfunctional (Figure 1). A class of proteins referred to as heat shock proteins (HSPs) function as molecular chaperons to refold metastable proteins, degrading damaged proteins, and synthesizing new proteins. During normal temperature conditions HSPs account for 5-10% of all proteins (Hu, 2022). During heat shock, expression of HSPs increases, suggesting increased mitochondrial activity and resulting increased ROS production. The statistical increase (Figure 3) in DCF fluorescence intensity at 521 nm emission between the control 37°C condition and heat shock 42°C indicates a possible causal relationship between the heat shock response and oxidative stress in L Cells (CRL-2648). Future experiments would include more specific heat shock conditions (37-50°C) to determine at which heat shock temperature oxidative stress is highest.

Sources

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- Kim, Hyeoncheol, and Xiang Xue. "Detection of Total Reactive Oxygen Species in Adherent Cells by 2',7'-Dichlorodihydrofluorescein Diacetate Staining." *Journal of visualized experiments : JoVE*, 160 10.3791/60682. 23 Jun. 2020, doi:10.3791/60682
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