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Quantification of Phenotypic Change Resulting in Sensitization of Primary Sensory Neurons due to Oxidative Stress

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Quantification of Phenotypic Change Resulting in Sensitization of Primary Sensory Neurons due to Oxidative Stress

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MOTIVATION AND SIGNIFICANCE

- c[1–3]
- When healthy, the IVD is mainly aneural and absorbs compression loads for spine motion and flexibility.[4,5]
- Age or injury can cause progressive degeneration of the IVD, and this can allow innervation by nociceptive (pain-sensing) neurons from the dorsal root ganglion (DRG) into the inner IVD.[6]
- Long-term exposure to pain-inducing stimuli can cause nociceptor sensitization, manifested as increased pain sensation in response to non-painful or painful stimuli.[7]
- Sensitization results in the nociceptors responding more strongly to a stimulus, and the activation energy required for response is reduced.[8]
- During IVD degeneration, inflammation and oxidative stress create excess reactive oxygen species (ROS) .[9]
- ROS such as superoxide may play a role in nociceptive signaling and lead to nociceptor sensitization.[8]
- ROS are involved in the chronic pain that results from nerve injury or inflammation in many contexts, and may be involved in discogenic pain as well.[10]
- **The focus of this study is to determine if long-term oxidative stress causes a change in the DRG nociceptive neuron phenotype related to sensitization. This would increase the expression of pain-related ion channels and lower the neuronal activation threshold.**

METHODS

DRG Harvest

1. DRGs dissected from sacrificed neonatal or adult rats.
2. Surrounding tissue removed and neuronal projections (neurites) trimmed.
   a) Some DRGs flash frozen and stored at -80°C for subsequent RNA isolation.
   b) Some DRGs and embedded in hydrogel for culture (see below).

DRG Culture

1. DRGs embedded in hydrogels consisting of type I collagen, laminin, and methacrylated hyaluronic acid (MAHA).
2. Cultured in 300 µL complete DRG media (Neurobasal media with 10% FBS, 1% Glutamax, 1% pen/strep, 2% B27, and 0.01% NGF).
3. ½ media changed every other day.
4. DRGs cultured for six days to allow neurite growth.
5. Imaged on day six using bright-field microscopy. (Figure 1, scale bar 500 µm)

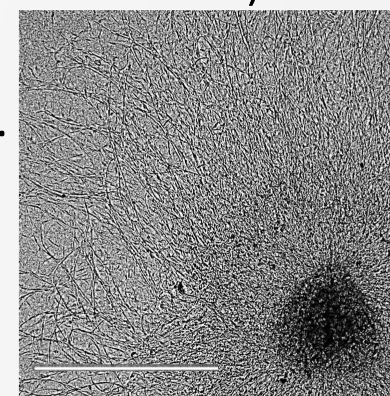

Figure 1. – Bright-field image of DRG after six days of culture in hydrogel.

METHODS

ROS Induction

1. DRGs embedded in hydrogels and cultured for 4 days in normal media.
2. DRGs were treated 3 times every 3 hours for 3 days with the ROS hydrogen peroxide (H_2O_2) and the intracellular antioxidant inhibitor L-BSO to induce oxidative stress or cultured in normal media as a control.
   - 100 µM H_2O_2
   - 100 µM H_2O_2 + 10 µM L-BSO
   - 200 µM H_2O_2
   - 200 µM H_2O_2 + 10 µM L-BSO
3. Separately, oxidative stress induction by culturing in different concentrations of oxygen was performed after 3 days of culture in normal media in normoxia. Normoxia culture is 21% O_2, 5% CO_2. Hypoxia is 3.5% O_2, 5% CO_2.
   - Normoxia
   - Hypoxia for 30 min every 3 hrs for 3 days, otherwise normoxia
   - Hypoxia
   - Normoxia for 30 min every 3 hrs for 3 days, otherwise hypoxia

Analysis:

1. ROS quantification using the fluorescence probe dihydroethidium (DHE).
   i. DRGs incubated in 20 µM DHE.
   ii. DRG gels embedded in cryo-medium and flash frozen in liquid nitrogen.
   iii. Frozen DRG blocks sectioned onto slides and immediately imaged on confocal microscope to detect fluorescence from ROS-reacted DHE.
   iv. Sections stained with DAPI for 3 minutes followed by re-imaging on the confocal to visualize cell nuclei.
2. DRG gels fixed, cryoembedded, sectioned, immunofluorescence for oxidative stress markers, and fluorescence intensity quantified using Image J (NIH):
   - 8-hydroxy-2'-deoxyguanosine (8-OHdG; RNA/DNA damage)
   - 4-hydroxylnonenal (lipid damage)
   - Carboxymethyl lysine (protein damage)

RNA Isolation

1. Flash frozen DRG samples homogenized and lysed using TRI Reagent.
2. Chloroform used to separate sample into three phases. A red organic phase (containing protein), an interphase (containing DNA), and an aqueous phase (containing RNA). (Figure 2)
3. Aqueous phase transferred to another vial.
4. RNA phase then precipitated into RNA pellet using isopropanol. (Figure 2)
5. RNA pellet was washed and resuspended in nuclease-free water for NanoDrop analysis.

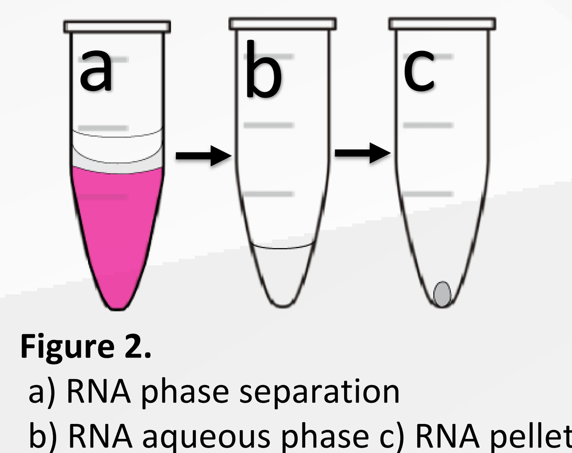

Figure 2.
a) RNA phase separation
b) RNA aqueous phase c) RNA pellet

RNA Isolation and cDNA Synthesis

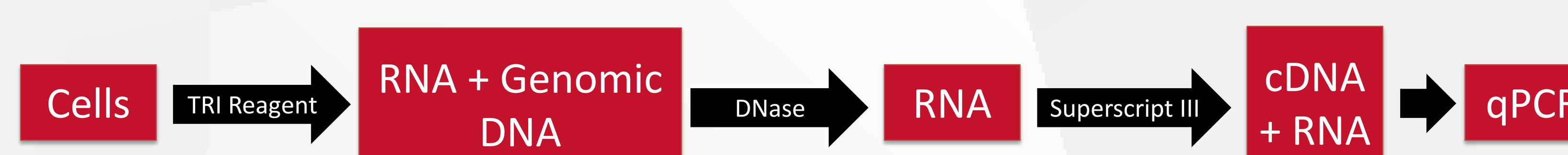

Figure 3. Overview of steps to prepare for qPCR

qPCR Design

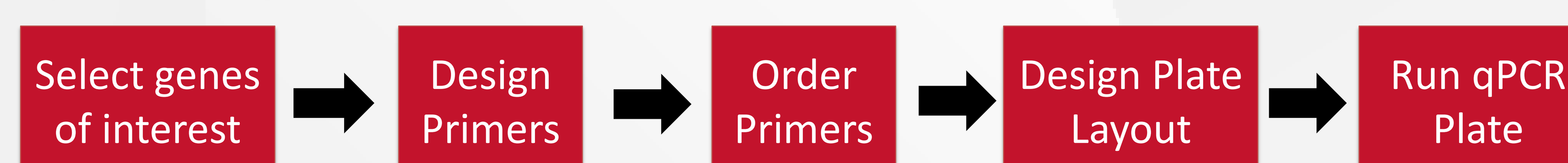

Figure 4. Overview of qPCR plate preparation.

RESULTS

RNA Isolation

The wavelength absorbance ratios measured by NanoDrop A_{260}/A_{280} & A_{260}/A_{230} indicate whether RNA quality is desirable to use for qPCR analysis. (Table 1 & Figure 5)

Sample ID	Nucleic Acid Conc. (ng/µl)	260/280	260/230
1	151.5	1.86	1.38
2	217.3	1.89	2
3	144.4	1.8	2.09

Table 1. NanoDrop results from RNA isolation

- An A_{260}/A_{280} of 1.8-2.1 indicates highly purified RNA, and an A_{260}/A_{230} near 2.0 is ideal, therefore samples 2 and 3 favorable for subsequent cDNA synthesis and qPCR analysis. (Figure 3)

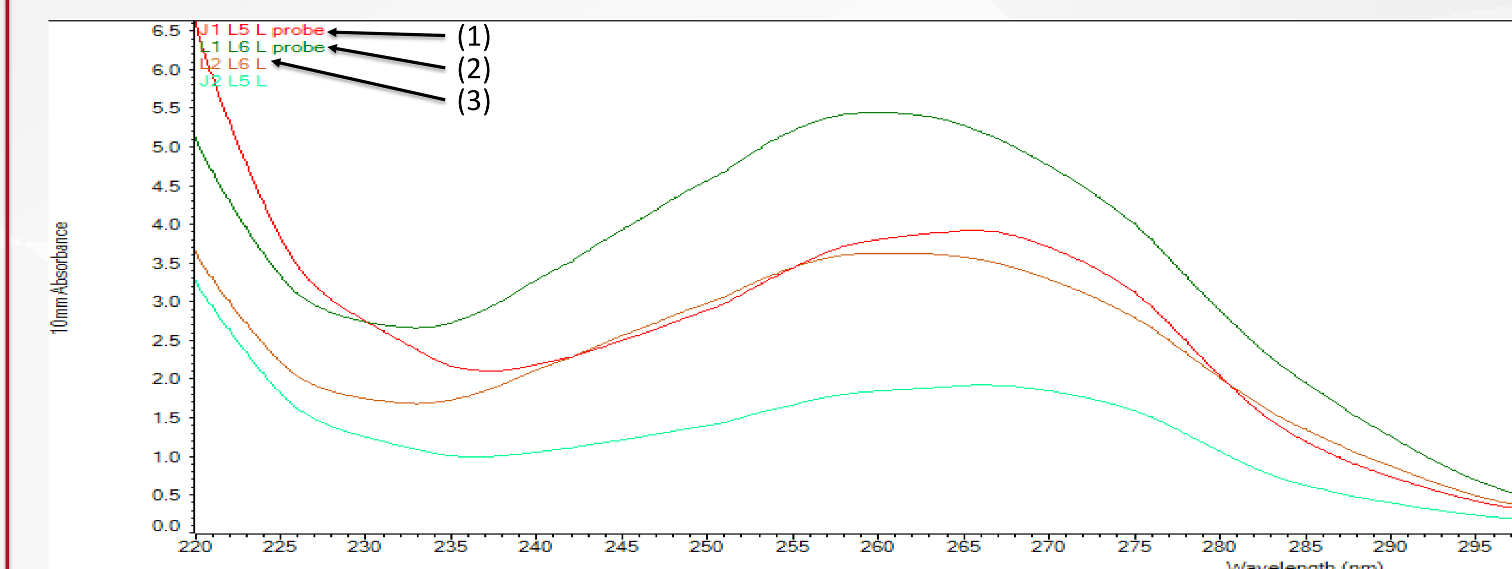

Figure 5. NanoDrop results from RNA isolation

ROS Induction

- 200 µM H_2O_2 + 10 µM L-BSO showed a significant difference in fluorescence intensity of the RNA/DNA damage marker 8-OHdG.
- Slight increase in 8-OHdG fluorescence in hypoxia-to-normoxia condition.

DISCUSSION AND FUTURE WORK

- Current ROS treatments cause some oxidative stress in DRG explants but not enough.
- We will move forward with optimizing additional analysis methods such as qPCR (Figure 4), Western blotting, and live calcium imagining.
- Future work will focus on increasing oxidative stress levels in DRGs to desirable levels while keeping the cells alive.
- Once oxidative stress inducing methods are established it will then be possible to determine if long-term oxidative stress causes a phenotypic change in DRG sensory neuron phenotype.

ACKNOWLEDGEMENTS

- Thank you to UCARE UNL for supporting and allowing me the opportunity to conduct this research.

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