ABSTRACT

Use of Mammalian Cell Culture Models to Determine the Role of OCM in Calcium Signaling

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Hearing loss is a prevalent problem worldwide. One of the critical aspects of hearing is the Ca^{2+} ion buffering system of the outer hair cells. It has been observed that there is an increase of Ca^{2+} levels after noise damage and during aging, and this can be associated with mitochondrial stress. We hypothesize that OCM will function as a buffer by helping the calcium related stress for the cells. We performed cell transfections, drug treatment, immunofluorescence, confocal imaging and analysis in order to qualitatively and quantitatively measure the cellular effects and potentially see the cellular mechanisms. We used cell culture because we can easily transfect the cells with plasmids of proteins found in the inner ear and we cannot directly isolate and manipulate outer hair cells. We specifically used Antimycin A and Thapsigargin because they are drugs that can be administered to cells to bring about mitochondrial stress that can lead to programmed cell death. By measuring the effects of these drugs on HeLa and HEK 293T cells transfected with GFP, OCM-GFP, and APV-OCM plasmids, and we observed how this stress affects the Ca^{2+} control and regulation. We wanted to explore how OCM affects cell survival through calcium signaling using the two drugs. In conclusion, we observed that OCM does not function as a mediator of mitochondrial stress under our specific conditions. Greater insight regarding these effects and cellular mechanisms will be helpful for further studies about inner ear mechanisms.

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USE OF MAMMALIAN CELL CULTURE MODELS TO DETERMINE THE ROLE OF OCM IN CALCIUM SIGNALING

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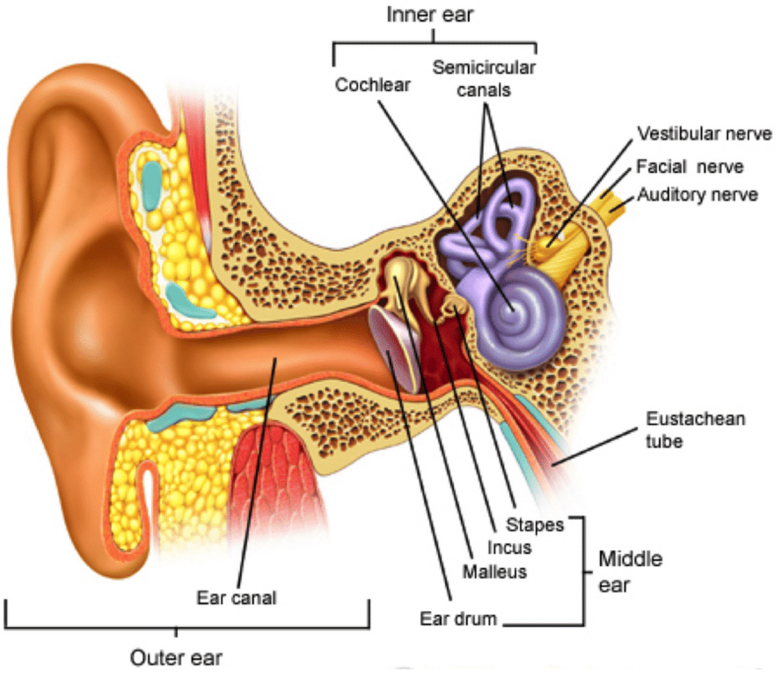
CHAPTER ONE

Introduction

Hearing loss

Hearing loss is a common sensory impairment that affects approximately 5.3% of the world's population (Gonzalez-Gonzalez 2017). Although some people are born with hearing loss, it can also develop at any age depending on factors such as noise trauma, ototoxicity, or age-related cochlear degeneration linked to common pathogenesis due to the formation of reactive oxygen species (ROS) (Gonzalez-Gonzalez 2017). Mitochondria are considered the main producer of ROS and it is observed that the oxidative stress caused by noise, ototoxicity, and cochlear degeneration can result in overproduction of ROS generation that can lead to hair cell death and thereby hearing loss (Brookes et al. 2004; Adam-Vizi and Starkov 2010; Feissner 2009). Exploring the process of apoptosis caused by mitochondrial stress involved in hearing loss is crucial in understanding and finding potential treatments.



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Auditory System

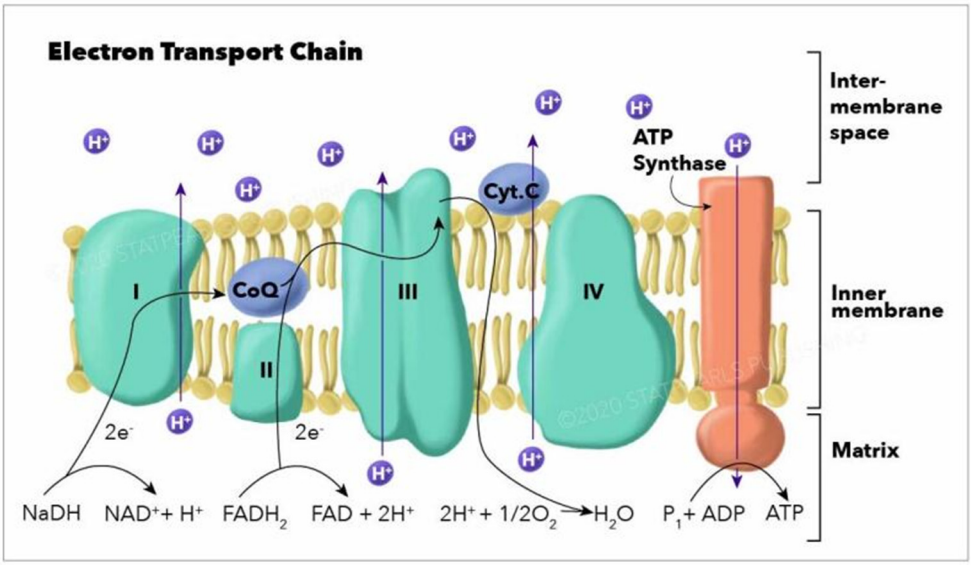
The auditory system consists of the outer ear, middle ear and the inner ear. The inner ear is composed of three fluid-filled compartments – the scala vestibuli, the scala media, and the scala tympani - which form a continuous cochlear duct (Slepecky 1996). The cochlea is separated from the scala tympani by the basilar membrane. Specifically, cochlea is responsible for mechanoelectrical transduction, which is the translation of mechanical signals into electrical impulses. The organ of Corti is located on the basilar membrane, containing the sensory hair cells (LeMasurler and Gillespie 2005). In the mammalian cochlea, this mechanoelectrical transduction occurs in two types of hair cells, the inner hair cells (IHCs) and outer hair cells (OHCs) (Fettiplace and Kim 2014). Prestin

(*Slc26a5*) is a voltage-sensitive motor protein that is primarily responsible for the contraction and relaxation of OHCs (Liberman et al. 2002).

There are a variety of different cell types that comprise the organ of Corti and each type plays a unique role. Pillar cells provide the structural support for the tunnel of Corti (Dallos et al. 1996). Dieter's and Hensen's cells maintain the location of OHCs (Pickles 2012). There are significantly more OHCs in the cochlea (3 rows) than there are IHCs (1 row) (Ashmore 2008). The OHCs and IHCs comprise less than 1 percent of cells belonging to the membranous labyrinth (Ranum et al. 2019). Firstly, IHCs are mostly connected with the afferents and function as the primary sensory cells that detect and relay acoustic information to the central nervous system (P Dallos 1992). On the other hand, OHCs are dominated by efferent nervous connections, and function as mechanical effectors that modulate the amplification of the traveling fluid waves (P Dallos 1992). OHCs actively amplify these vibrations by contracting and relaxing in response to mechanical stimulation. It has been observed that the loss of OHCs results in hearing loss (Ryan and Dallos 1975).

When entering the ear, the sound vibrations travel through the auditory canal and the oscillations of the air pressure vibrate the eardrum which causes ossicles to vibrate in the middle ear. These waves reach the cochlea with sound pressure fluctuations and the vibrations are transformed at the cochlea into fluid distortions (Fettiplace 2006). Eventually, the hair cells of the Organ of Corti are stimulated by the vibrations of the basilar membranes, initiating the mechanotransduction by deflecting the hair bundles (LeMasurier and Gillespie 2005). The "hairs" are stereocilia, which are composed of

actin filaments and are arrayed by increasing height. The basilar-membrane oscillations cause the basal tapering of the stereocilia, which opens the mechanosensitive transduction channels which allows the influx of K+ ions into the hair cells. This depolarization opens Ca^{2+} voltage-gated channels near the basolateral synapses, stimulating the neurotransmitter release to initiate the signal propagation to the afferent neurons (Fettiplace and Ricci 2006).



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Mitochondria

In eukaryotic cells, mitochondria play an important role by providing energy in the form of ATP (Borutaite 2010). Mitochondria is also involved in calcium signaling (Contreras et al. 2010) and regulation of autophagy (Scherz-Shouval and Elazar 2007). Further, mitochondria are involved in fatty acid metabolism, calcium signaling, cellular metabolism, steroid synthesis, heme production, apoptosis.

Mitochondria are the main site of ATP synthesis in aerobic cells (Sherratt 1991). Mitochondria have a matrix space that is enclosed by an inner membrane. The inner membrane contains four complexes of the electron transport chain, ATP synthase, and electron transporters ubiquinone and cytochrome complex (Zhao et al. 2019). They also have a relatively permeable outer membrane and an intermembrane space. The integrity

of the mitochondrion is essential for ATP synthesis (Sherratt 1991). Specifically, the generation of a proton gradient across the inner membrane allows for the electron flow. The energy from this flow is accumulated and used by the ATP synthase to produce ATP. The electron flow is coupled with the generation of a proton gradient across the inner membrane and the energy accumulated in the proton gradient is used by ATP synthase to produce ATP (Zhao et al. 2019).

Mitochondria are important in apoptotic pathways. Cells on the pathway toward apoptosis (programmed cells death) display a decrease in mitochondrial membrane potential and increased mitochondrial swelling (De Giorgi et al. 2002). These changes might follow the opening of mitochondrial permeability transition pores (mPTP), which are high-conductance proteinaceous channels in the inner mitochondrial membrane. It has been discovered that when the cell undergoes oxidative stress, cytosolic protein Bax translocates to the mitochondria, which functions as a prerequisite for cytochrome complex release from the permeability transition pores, which in turn leads to the rupture of outer mitochondrial membrane (De Giorgi et al. 2002).

Apoptosis occurs at a single cell level, and is important for maintaining homeostasis in multicellular organisms. Apoptosis can be triggered by factors such as receptor-mediated signals, withdrawals of growth factors, damage to DNA, or excess oxidative stress caused by the mitochondria (Kannan and Jain 2000). During mitochondrial dysfunction, the mitochondria releases proapoptotic factors such as cytochrome complex and protease-activating factor-1(APAF-1), which form complexes

in the cytosol to activate downstream caspases that lead to apoptosis (Kannan and Jain 2000).

ROS Production and Inner Ear

In hair cells, mitochondria play a role as regulators for mechanotransduction, affecting the function and dysfunction of hair cells. Specifically, they are involved with ATP production, regulation of calcium dynamics and regulation of redox balance. They are located next to the cuticular plate alongside the subsurface cisternae in the OHCs (Weaver and Schweitzer 1994; Fettiplace and Ricci 2006).

Reactive oxygen species (ROS) play a significant role in cell signaling and are produced in the mammalian mitochondria as a result of the dysfunction in the electron transport chain, and is an important sign for oxidative stress within the cell (Murphy 2009). In the normally functioning electron transport chain, some electrons are used to generate ROS. ROS plays an important role in maintaining cell homeostasis and signaling (Zhao et al. 2019).

In healthy conditions, the ROS is metabolized and scavenged by endogenous antioxidant mechanisms to balance cell homeostasis, but in aging conditions, this balance can be ineffectively regulated and thus create oxidative stress (Gonzalez-Gonzalez 2017). Increased levels of mitochondrial Ca^{2+} is associated with mitochondrial oxidation and cytoplasmic ROS. Increase in mitochondrial ROS is regulated by Ca^{2+} uptake (Esterberg et al. 2016).

Regarding most cell types, mitochondria are considered the main producer of ROS and it is observed that Ca^{2+} uptake, transmembrane potential, and ROS generation

in mitochondria are closely associated with hearing loss which leads to hair cell death (Brookes et al. 2004; Adam-Vizi and Starkov 2010; Feissner 2009). Specifically, dysregulation of Ca^{2+} is responsible for ROS accumulation and consequently apoptosis.

Ca^{2+} main role in the mitochondria is the promotion of ATP synthesis through physiological Ca^{2+} signals which adjusts the ATP production to cellular demand. When there is overproduction of ROS, it causes the influx of Ca^{2+} into the mitochondria and disrupts the normal function (Adam-Vizi and Starkov 2010).

Mutations in genes linked to mitochondrial function and ROS regulation have been associated with hearing loss (Gonzalez-Gonzalez 2017). Noise overstimulation and ototoxic drugs can result in excess ROS production in OHCs that can lead to genetic and cellular alterations which cause cellular dysfunctions such as lipid peroxidation, polysaccharide depolymerization, nucleic acid disruption, oxidation of sulfhydryl groups, and enzyme inactivation, consequently leading to permanent cochlear degeneration (Gonzalez-Gonzalez 2017). Furthermore, this can create fused hair cell stereocilia and degeneration of supporting structures and nerve fibers; degenerative changes are also observed in the stria vascularis (Shi and Nuttall 2003). Additionally, there also is the possibility of ROS overproduction causing the deletion of hair mtDNA which results in mitochondrial dysregulation and apoptosis of hair cells (Gonzalez-Gonzalez 2017).

Ca^{2+} *Signaling in the Inner Ear*

Cells in the inner ear need a mechanism to be able to adjust to the physical and biochemical environmental changes that result from such as noise trauma or ototoxicity in order to maintain intracellular homeostasis. Ca^{2+} signaling is the main method of

communication. Ca^{2+} signaling is important for relaying auditory stimuli to higher order brain structures (Jiang et al. 1994). The OHCs control the localization and timing of Ca^{2+} signaling by regulating Ca2+ influx and efflux through a collaboration of plasma membrane ion channels and transporters and internal storage compartments.

Soluble, mobile Ca^{2+} binding proteins (CaBPs), such as **α**-parvalbumin (aPV) and Oncomodulin (OCM), aka β-parvalbumin, that serve as the primary Ca^{2+} buffers for regulating intracellular calcium levels in IHCs and OHCs, respectively (Climer et al. 2019). Ca^{2+} - binding proteins (CaBPs) are important regulators of Ca^{2+} homeostasis controlling the influx and efflux of Ca^{2+} during mechanotransduction. aPV and OCM both belong to the EF-hand calcium-binding protein family and have similarities in structural and functional characteristics (Climer et al. 2019).

During mechanotransduction, the Ca^{2+} enters the OHC as the stereocilia deflects (Beurg et al. 2010; Fettiplace and Kim 2014). Ca^{2+} influx further activates the Ca^{2+} - activated potassium channels (SK2) through the cholinergic ionotropic receptors (nAChRs) and this occurs at the basal side of the OHCs (Dulon et al. 1998). This leads to the hyperpolarization of OHCs in a calmodulin (CaM)-dependent fashion (Xia et al. 1998). Calcium induced calcium release (CICR) occurs in the OHCs when the influx of Ca^{2+} waves stimulate the release of Ca2+ from ER-like sub-surface cisternae (SSC) and mitochondrial storage (Ashmore 2008). As a result, signals generated from the influx of Ca^{2+} ions are intensified (Evans et al. 2000). The SERCA family of proteins are the major transporter of Ca2+ into lumen of the ER (Mella et al. 2003). Ryanodine receptors (RyRs) and 1,4,5-trisphosphate receptors (IP3Rs) release Ca^{2+} from the ER (Franzini-Armstrong

and Protasi 1997). As noted, ER and mitochondria play crucial roles in this calcium release process.

Oncomodulin

Oncomodulin (OCM) belongs to the parvalbumin family and functions as a small EF-hand CaBP of approximately 12kDa (Climer et al. 2019). In addition to OCM, the mammalian inner ear has other major EF-hand CaBPs, such as α-parvalbumin (aPV), calbindins (CB-D28k), calretinin (CB-D29k), calmodulin, and S100 proteins (Climer et al. 2019). Specifically, mammalian OCM is the β isoform of parvalbumin and displays a limited expression pattern in mammals: restricted mostly to subsets of sensory hair cells in the inner ear (Climer et al. 2019). OCM is the major CaBP expressed in mammalian cochlear OHCs and is mainly localized to the lateral cell membrane. Of the major EF-hand CaBPs, only deletion of *Ocm* leads to hearing loss in adult mice (Tong et al. 2016). Recently, a new CaBP, sorcin, has been discovered in OHCs. Sorcin (*Sri*) is located at the subsurface cisternae of OHCs (Ranum et al. 2019). In cardiac myocytes, sorcin facilitates Ca^{2+} dependent inhibition of ryanodine receptor (RyR) channels. This is important for calcium-mediated excitation-contraction coupling, implying that the presence of sorcin leads to tight regulation of calcium in OHCs. Additionally, *Ocm* and *Sri* are the top two genes that transcriptionally identify OHCs (Farrell et al. 2003). Since OCM is a Ca^{2+} buffer preferentially localized to the lateral membranes near subsurface cisternae in OHCs, OCM may work in the same cellular compartments.

Antimycin A(AMA)

Antimycin A (AMA) is an inhibitor of succinate oxidase, NADH oxidase and mitochondrial electron transport chain between cytochrome b and c (Tzung et al. 2001). Treatment with AMA results in apoptosis, loss of mitochondrial membrane potential, increased ROS levels, increase in intracellular O2 and depletion of GSH in As4.1 juxtaglomerular cells (Han et al. 2009). Previous studies have also demonstrated that AMA effectively produces ROS, depletes GSH content and inhibits cell growth in HeLa cells throughout apoptosis (Han et al. 2008). AMA is primarily composed of antimycin A1 and A3, which are derived from *Streptomyces kitazawensis*. As AMA directly acts on the mitochondria electron transport chain, it leads to the collapse of proton gradient across the mitochondrial membrane which results in the loss of membrane potential and increase in ROS levels (Tzung et al. 2001).

There is a strong possibility that opening of mitochondrial permeability transition pore by the presence of ROS leads to the release of proapoptotic molecules such as cytochrome c into the cytoplasm (Han et al. 2009). AMA induces mitochondrial stress by inhibiting complex 3 of ETC at the site of cytochrome c reductase (CoQH2-cytochrome c reductase). This prevents the electron flow through ETC and leads to the accumulation of O2- in the mitochondria, the interruption of ATP synthesis and induction of apoptosis.

Thapsigargin (Tg)

Thapsigargin (Tg) is a selective and irreversible inhibitor of SERCA (the ER Ca^{2+} pump) and induces apoptosis through prolonged activation of the unfolded protein

response (UPR) (Thastrup et al. 1990). Based on the previous studies, Tg induces apoptosis via death receptor 5 and caspase –8 in cancer cell lines (LNCaP prostate- and HCT116 colon cancer cells). Apoptosis is induced by excessive ER stress that leads to UPR. “ER stress” refers to the failure of folding synthesized proteins and therefore the accumulation of unfolded proteins in the ER (Lindner et al. 2020).

The dynamic of mitochondrial fission and fusion plays an essential role in mitochondrial morphology (Bereiter-Hahn and Vöth 1994; Sesaki and Jensen 1999). Mitochondrial fragmentation is a common phenomenon during apoptosis, and it has been found that proapoptotic protein Bax are localized to the constriction areas of the mitochondria during apoptosis (Karbowski et al. 2002). Specifically, treatment with Tg has been shown to induce two mitochondrial phases: The first phase of fragmentation was transient and reversible, associated with increased intracellular Ca2+ levels and Ca2+ influx into mitochondria, and the second phase was non-reversible and led to apoptosis as a result of prolonged incubation with Tg (Hom et al. 2007). This suggests that Ca^{2+} plays a significant role in mitochondrial morphology which determines life or death of cellular responses (Hom et al. 2007).

It is important to note that Ca^{2+} is an essential regulator of ATP generation, redox control and apoptosis (Brookes et al. 2004). Upon treatment with Tg, calcium released by the ER floods the cytosol and is taken up by the mitochondria (Brookes et al. 2004). The increased mitochondrial Ca2+ results in mitochondrial fragmentation suggesting a link between calcium and Ca2+ fission and fusion processes (Hom et al. 2007)**.** When the cell fails to repair the mitochondrial fragmentation through its saving cellular mechanisms,

the cell undergoes apoptosis. Prolonged treatment with Tg leads to apoptosis as a result of the loss of ER Ca^{2+} (Jiang et al. 1994; Kaneko and Tsukamoto 1994).

CHAPTER TWO

Materials and Methods

Cell Culture and Reagents

HeLa and Human Embryonic Kidney (HEK293T) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS, Bio-techne) at 37°C in a 5% CO_2 incubator. Transfections were performed using Lipofectamine 3000 (Thermo Fisher Scientific) in Opti-Mem reduced serum media (Thermo Fisher) according to manufacturer's protocol. HeLa and HEK293T cells were plated on Nunclon Delta Surface plates (Thermo Fisher Scientific) and transfected the following day at 60% confluency. 96-well plates were used for MTS assays. Thapsigargin (Tg, Invitrogen) and Antimycin A from *Streptomyces* sp. (AMA, Sigma-Aldrich) stocks were dissolved in DMSO (Sigma-Aldrich).

MTS Assay

MTS Assay is commonly used to measure cell proliferation and to analyze cytotoxic compounds such as toxic agents and anticancer drugs. The protocol is based on the reduction of tetrazolium compounds that generate a formazan dye soluble in cell culture media. The formazan dye is quantified by measuring the absorbance at 490-500 nm.

The MTS Assay Kit (Abcam) was used to determine the LD_{50} for AMA and Tg in HEK and HeLa cells. 96-well cell culture plates were seeded with HEK or HeLa so that they would reach 90% confluency on the day of the MTS read. Each plate received a range of doses from 0.78 uM - 200 uM AMA or 0.10 uM - 80 uM Tg as well as a row of control (vehicle only) and 20% DMSO death control. Drugs were diluted in FluoroBrite DMEM Media (Gibco) supplemented with 10% FBS and 10 mM L-glutamine.

A dose-response model pre-programmed into Prism 8 software was fit to the data and used to determine LD_{50} concentrations. For survival assays, cells were incubated with the MTS reagent for 2 hours at 37°C according to manufacturer's protocols. 96-well plates were shaken briefly. An xMark Microplate Absorbance Spectrophotometer (BioRad) was used to quantify colorimetric change at 490 nm. 8 replicates were measured for each treatment group, but wells on the edge of the plate were excluded from analysis. OD490 measurements were input into Prism 8, normalized so that 0% was set to the average of the 20% DMSO positive death control replicates and 100% was set to the average of the untreated control replicates.

Immunocytochemistry

24-hours post transfection, HEK293T and HeLa cells were administered AMA and/ or Tg for 24 hours (21.17uM for Tg, 72.20uM for AMA). For immunocytochemistry, cells were plated on 15 mm round coverslips (Electron Microscopy Sciences). For HEK cells, coverslips were pre-coated with Poly-L-Lysine (Sigma Aldrich). Cells were fixed with 4% paraformaldehyde (in PBS) for 10 minutes, neutralized with 50 mM NH_4Cl for 5 minutes, permeabilized with 1.0% Triton X-100 in PBS for 15 minutes and blocked with 1% BSA in PBS for 1 hour. The following primary antibodies (diluted in 0.1% BSA) were incubated overnight: anti-Bax (ThermoFisher # MA5-14003 1:50), anti-TOM20 (ThermoFisher # MA5-34964 1:100), anti-EndoG (Sigma SAB3500213 # 1:100), COX4 (CST #4D11-B3-E8 1:200), anti-Nrf2 (CST #12721S 1:50)

Species appropriate Alexa Flour (ThermoFisher) or Northern Lights (R&D Systems) conjugated secondary antibodies (diluted in 0.1% BSA 1:200) were incubated for 1 hour in the dark. Coverslips were mounted using ProLong Gold Anti-Fade (ThermoFisher) and left to cure overnight in the dark before imaging.

Confocal Microscopy and Statistical Analysis

Zeiss LSM800 with Airyscan confocal microscope with 63x Plan Apo (1.4N.A.) oil objective was used for imaging. For semi-quantitative analysis of immunohistochemistry, FIJI software was used to quantify the fluorescence for GFP-tagged proteins and Nrf2. Z stacks of 0.5 micron increments at total slice thickness of 5 micron were used for quantification. Region of interests (ROI) for nuclear regions were measured by using the DAPI channel to circle the nuclei. Cytoplasmic and whole cell ROIs were measured using Nrf2 to identify the plasma membrane. The cytoplasmic(c) and nuclear(n) measurements were calculated into c/n ratios and were graphed for comparisons using the Prism 8 software.

CHAPTER THREE

Results

When working with the effect of drug incubation in cell culture, it is important to describe and measure cytotoxicity. Commonly, LD_{50}, Lethal Dosage 50 is used to obtain the in vivo toxicity of a new agent. LD_{50} can be defined as the dose that kills half of the tested population. Within cell culture, it is the concentration of a drug that kills half of the tested cells in culture. Based on published data, it was predicted that approximately 50uM of AMA would be the measurement for LD_{50} (Han et al. 2009). Using our MTS assay, we calculated the LD_{50} for AMA and Tg in both HEK and HeLa cells: for AMA it was 9.427 uM and 58.23 uM for HEK and HeLa, respectively (**Figure 1)**. For Tg, the LD_{50} was found to be 15.94 uM and 21.17 uM for HEK and HeLa, respectively. We calculated higher LD_{50} of AMA and Tg for HeLa cells compared to HEK cells, this might be because the cancerous cells have a more resistant nature for survivability compared to non-cancerous cells. When we carried out the MTS assays, HEK cells reacted according to the LD50 results, experiencing around 60-70 percent cell death with the calculated dosages **(Figure 2)**. We can see that APV transfected cells had the highest cell survival rate, and OCM had the highest cell survival rate with the drug treatments, and this could be because OCM as a calcium buffer is relieving mitochondrial stress and thereby lowering the rate of apoptosis in the cells, but the difference was not significant.

On the other hand, HeLa cells did not respond to the LD50 dosages of AMA and Tg as expected. This might be because of the inaccuracies that arose from the protocol

and timeline of transfection experiments. This shows that AMA dosage needs to be higher in HeLa transfection experiments.

HeLa cells were treated with Tg and AMA for 24 hours individually or combined because we wanted to observe its individual effects and test whether the combined treatment results in higher levels of oxidative stress. Afterward, we looked for hallmarks of apoptosis such as localization and expression of pro-apoptotic Bax and mitochondrial marker, TOM20.

Under normal conditions, Bax resides in the cytoplasm. However, with induction of apoptosis, Bax will tether to the mitochondrial membrane, where TOM20, a membrane protein localized in the outer membrane of the mitochondria, is localized. Therefore, co-localization of Bax and TOM20 should be a hallmark of apoptosis. We noticed this about the apparent abundance of TOM 20 across treatments, we noticed this about Bax across treatments (**Figure 3**). We did see that they co-localize. Co-localization was not quantified but didn't appear to change dramatically based on the different treatments. The overall morphology of the mitochondrial network did appear to change based on the treatment groups. Based on the confocal images, we noticed the apparent abundance of TOM 20 and Bax across treatments that seemed to colocalize. This is suggested by the yellow color in the merge. Co-localization was not quantified but didn't appear to change dramatically based on the different treatments. The overall morphology of the mitochondrial network did appear to change based on the treatment groups. Specifically, based on the apparent TOM20 expression, the mitochondria seems to have spread out under AMA treatment, and all drug treatment groups had lesser TOM 20 expression

compared to the control. This was the same for Bax. This could mean less amount of mitochondria due to apoptosis under cell stress, or it could also mean that the drug treatment. Based on the smaller and fewer DAPI expressions under drug treatment compared to control, we also think the drugs negatively affected the overall cell health. However, we cannot know whether Bax is on the mitochondrial membrane or nearby in the cytosol due the limitations in resolution.

Next, we investigated the expression and localization of Nrf2, a transcription factor that regulates antioxidant responses (Li et al. 2018). It is localized in the cytoplasm and under oxidative stress, it translocated into the nucleus to activate the antioxidant response element (ARE) (Li et al., 2018). Nrf2 localization was measured and the mean cytoplasmic fluorescence intensity over the mean nuclear fluorescence intensity was calculated (c/n ratio). The lower the c/n ratio, the more localized the protein is to the nucleus. Under the untreated conditions, the transfection rate seemed similar across plasmids (**Figure 4**). However, saw that there is a decrease in apparent expression of Nrf2 expression in OCM transfected cells. This might be because OCM is functioning to decrease the mitochondrial stress. We speculate that the reason why these untreated cells have Nrf2 expression is because the cells are already in cellular stress due to the transfection.

When the transfected HeLa cells were treated with Tg, APV decreased in apparent Nrf2 expression (**Figure 5**). This could suggest that AMA somehow lessens the mitochondrial stress after transfection. On the other hand, the GFP-only transfected cells seem to show increased expression of Nrf2. In order to confirm these results, we will

have to make more replicates along with doing a condition of untransfected and untreated cell control.

When the transfected HeLa cells were treated with AMA, both GFP-only transfected cells showed less apparent expression of Nrf2 and APV transfected cells showed increased expression (**Figure 6**). Overall, the GFP expression seemed lower on all three plasmid conditions. We also saw that there were intense fluorescent balls in the AMA treated APV transfected cellsThese could represent autophagic clusters or apoptotic bodies. One way to confirm its identity would be to label them with autophagy markers in future experiments.

Lastly, we also looked at the combined effect of AMA and Tg on transfected cells because we wanted to see whether Tg and AMA would work together to intensify or lessen oxidative stress (**Figure 7**). Based on the images, we saw that the combination of AMA and Tg treatment decreased the apparent expression of Nrf2 for GFP-only and APV transfected cells, where for OCM transfected cells oppositely had higher expression. Another interesting detail is that compared to the untreated groups, the combined group seemed to have fluorescence located in the nucleus, especially in the OCM transfected cells. We can see in the merge that the nuclear fluorescence of Nrf2 expression caused the image to show a bright area at the center.

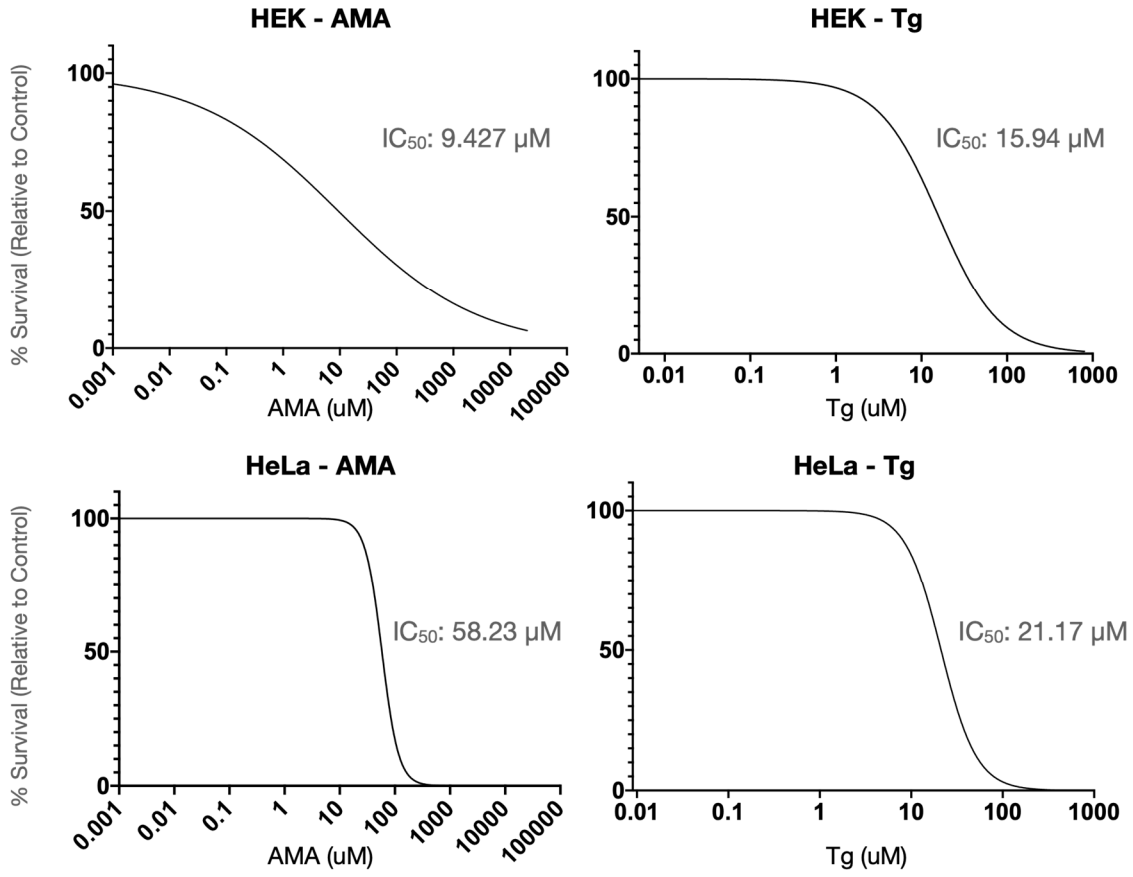

Figure 1. LD_{50} graphs of both inhibitors used; AMA (left) and Tg (right) in HEK (top) and HeLa (bottom) cells.

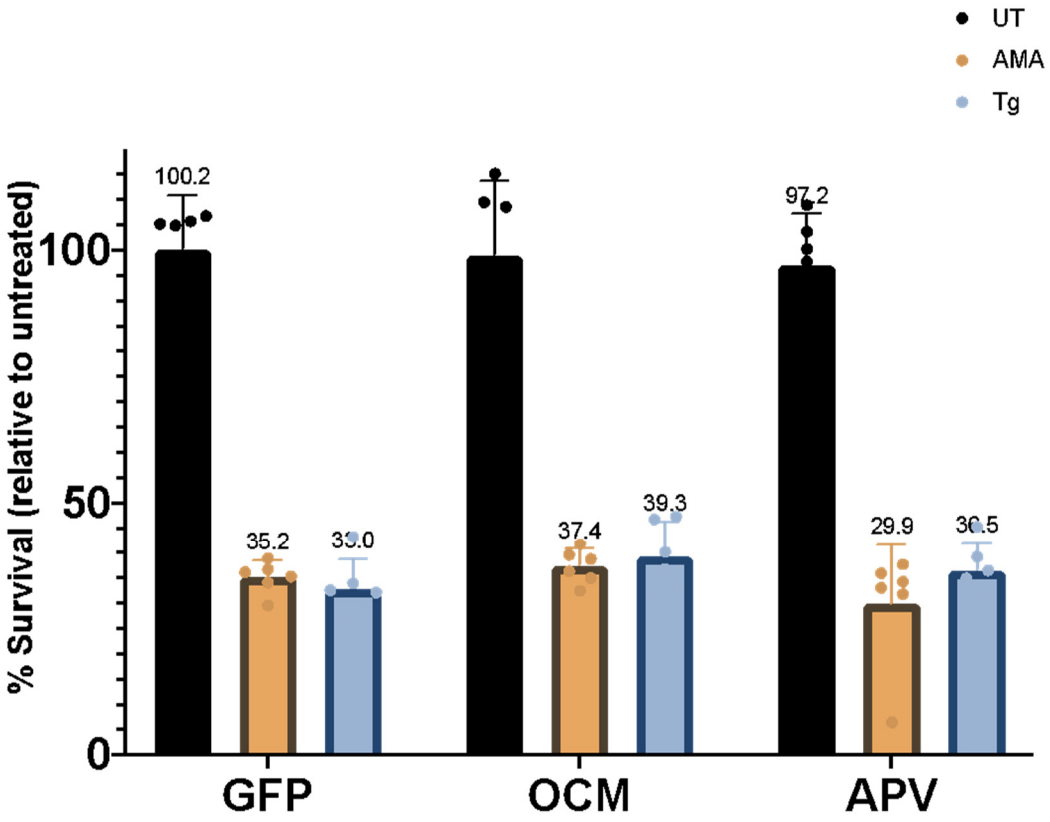

Figure 2. Treatment with AMA and TG on HEK cells resulted in approximately 60-70% decrease of cell survival. The difference between AMA and TG in terms of cell survival was insignificant.

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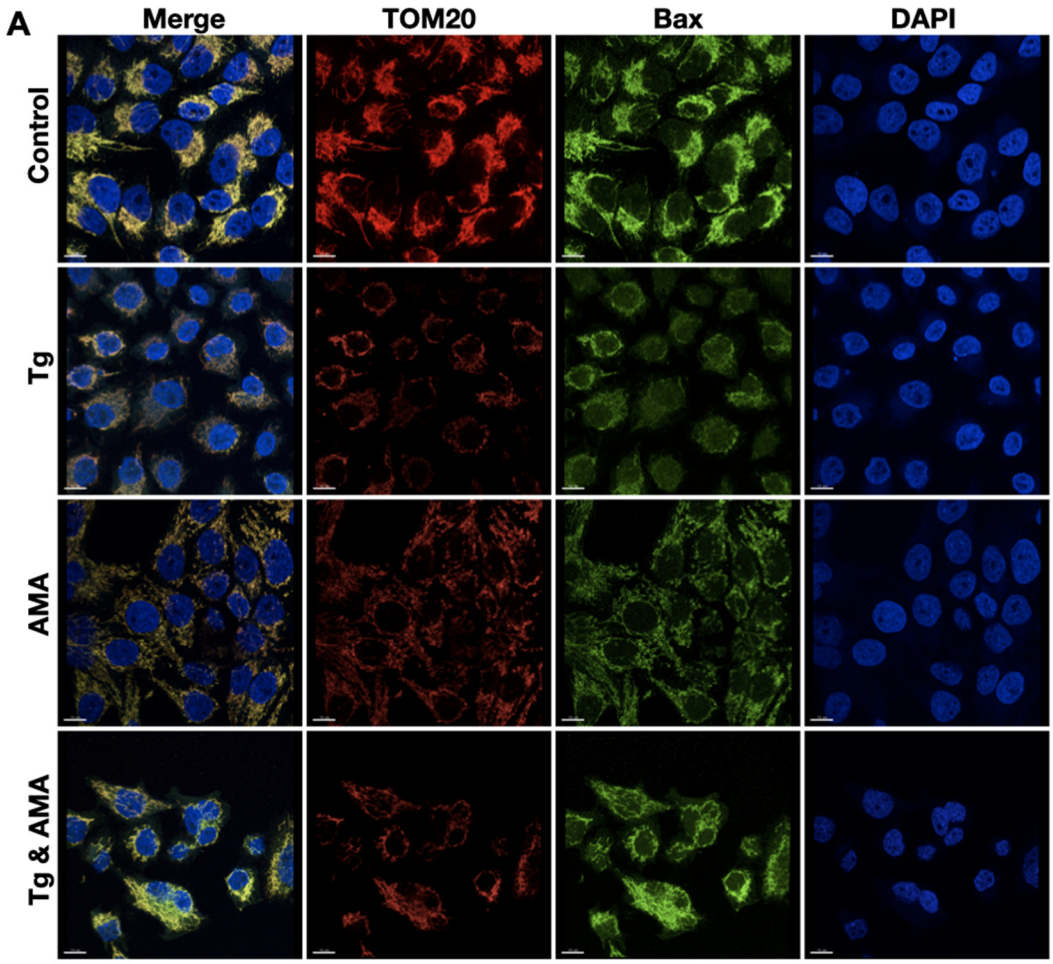

Figure 3. Confocal images of HeLa cells treated with control, Tg, AMA, or Tg & AMA. TOM20 (red) was used as a mitochondrial marker. Bax (green) was used as an apoptotic marker. DAPI shows nuclei.

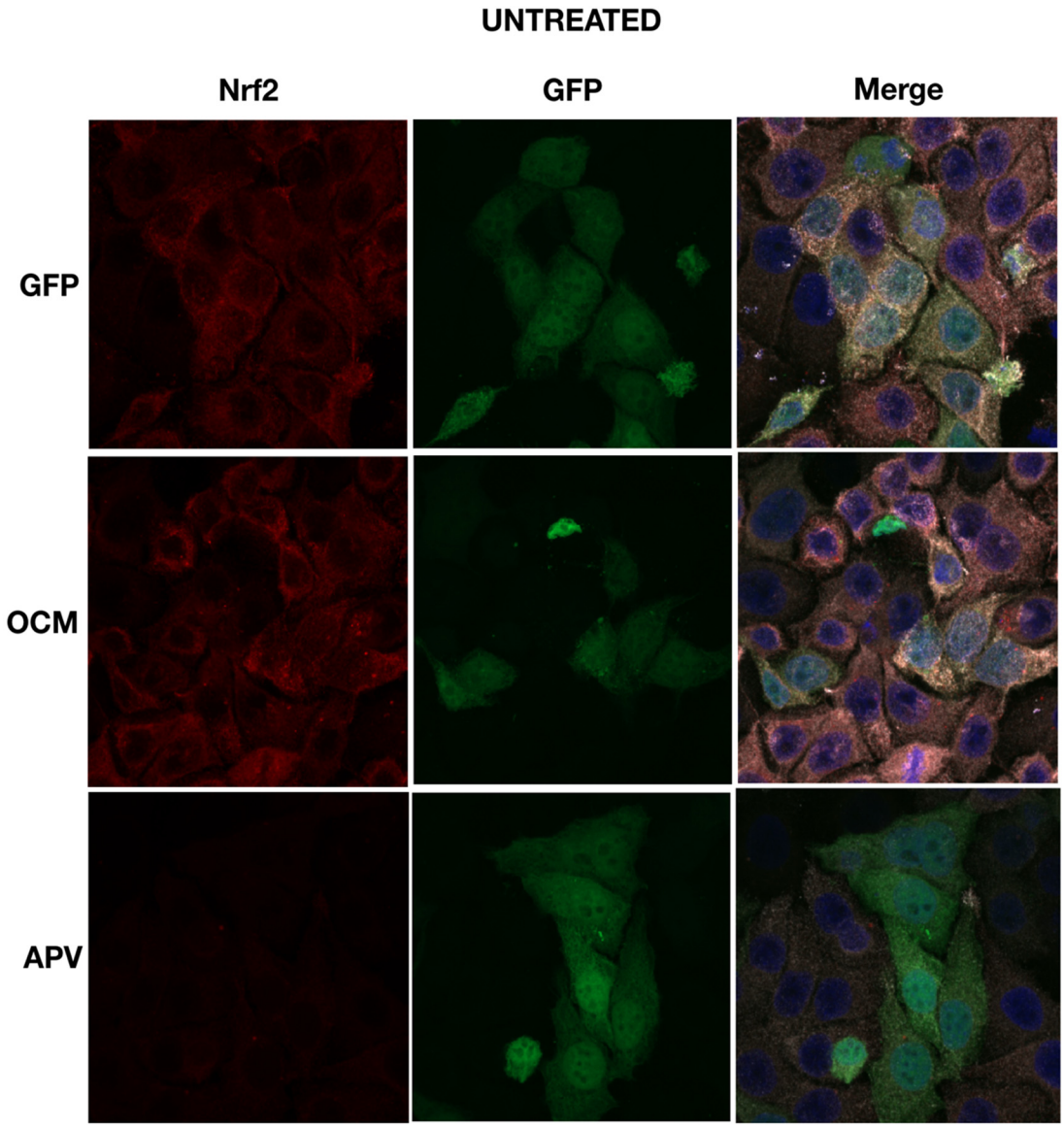

Figure 4. When the HeLa cells were not treated with any drugs, the transfection rate was the highest. Qualitatively speaking, the GFP transfected cells also expressed Nrf2 proteins. These images were used as controls for the experiment.

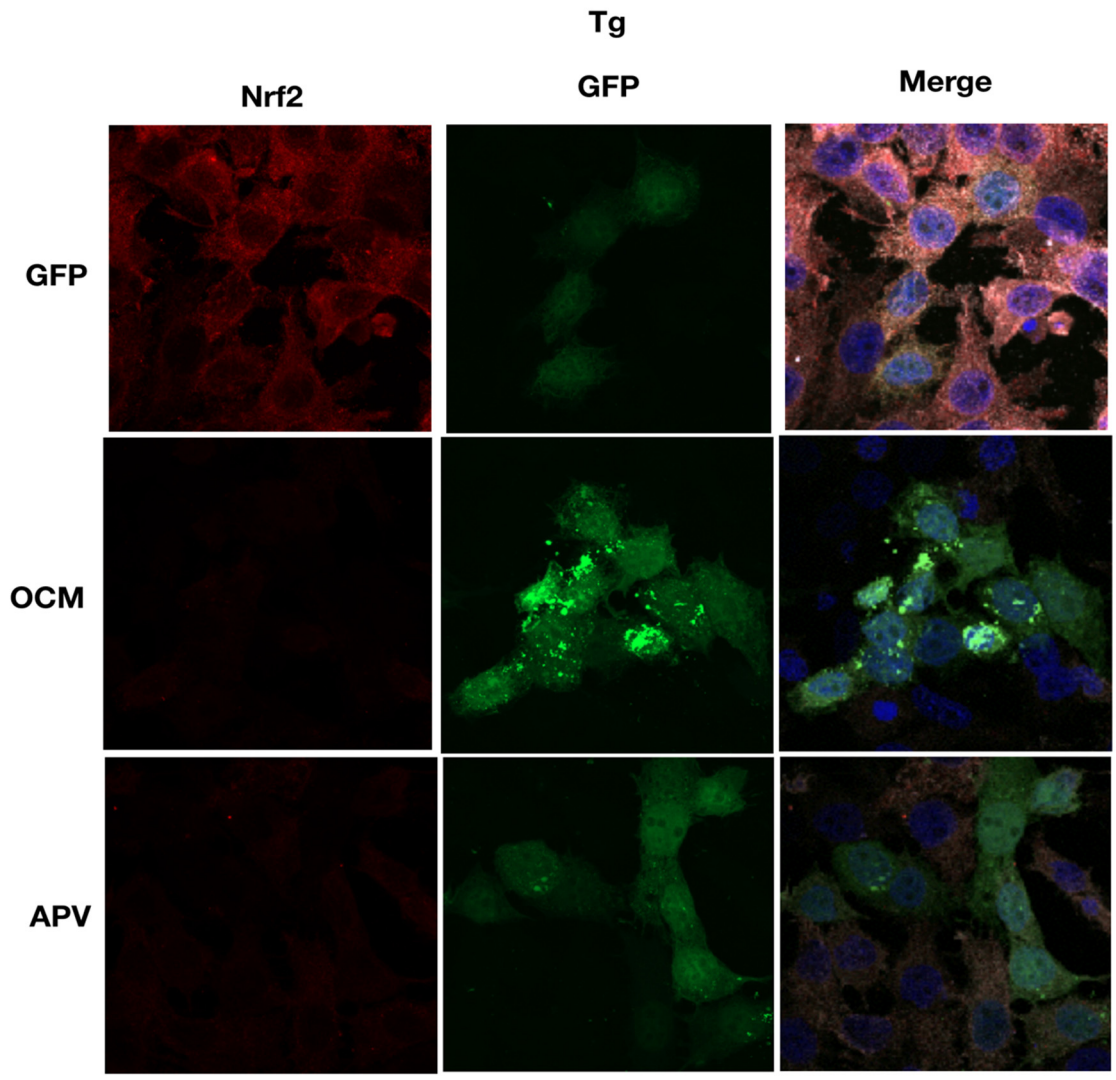

Figure 5. Compared to the untreated cells, the Tg treated cells had lower transfection rates due to cell-death.

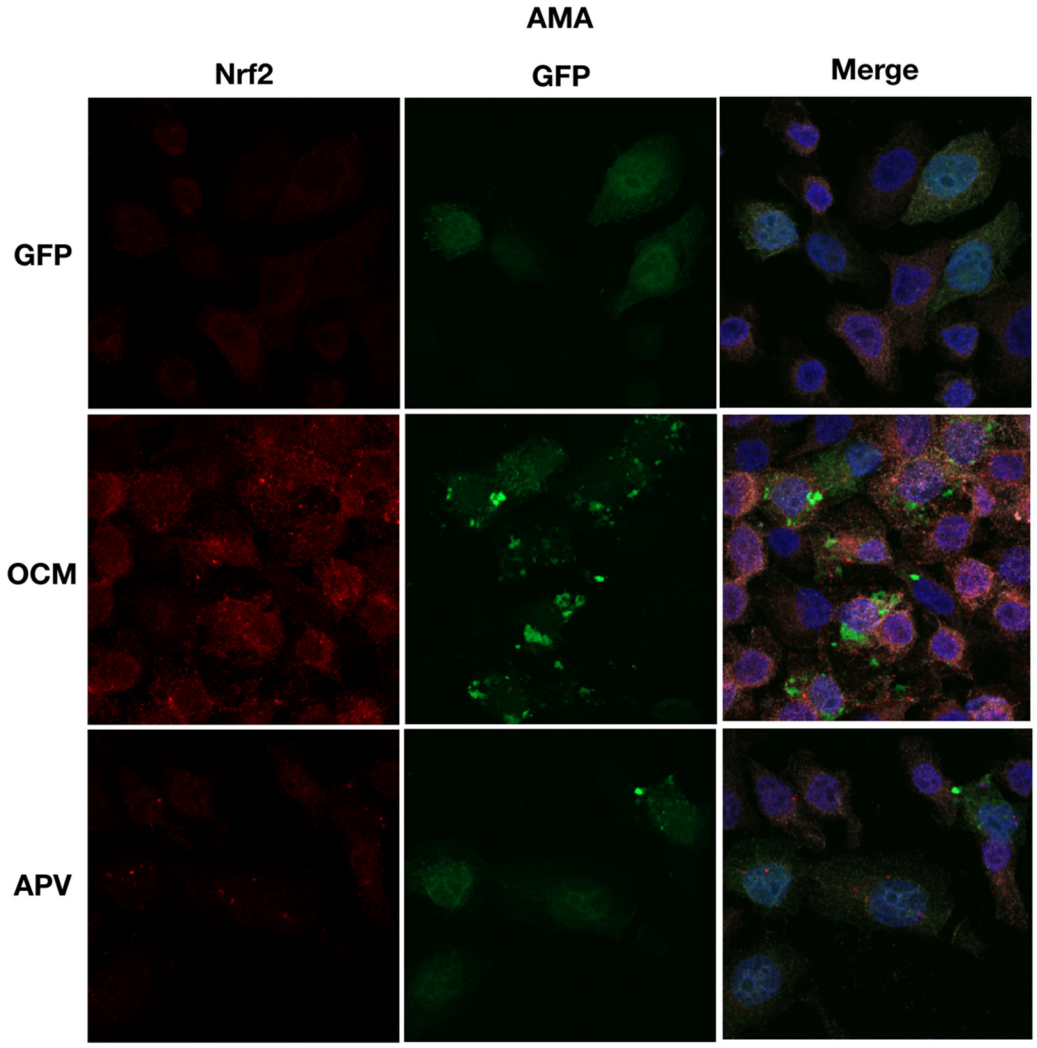

Figure 6. Compared to the untreated cells, the AMA treated cells had lower transfection rates due to cell-death.

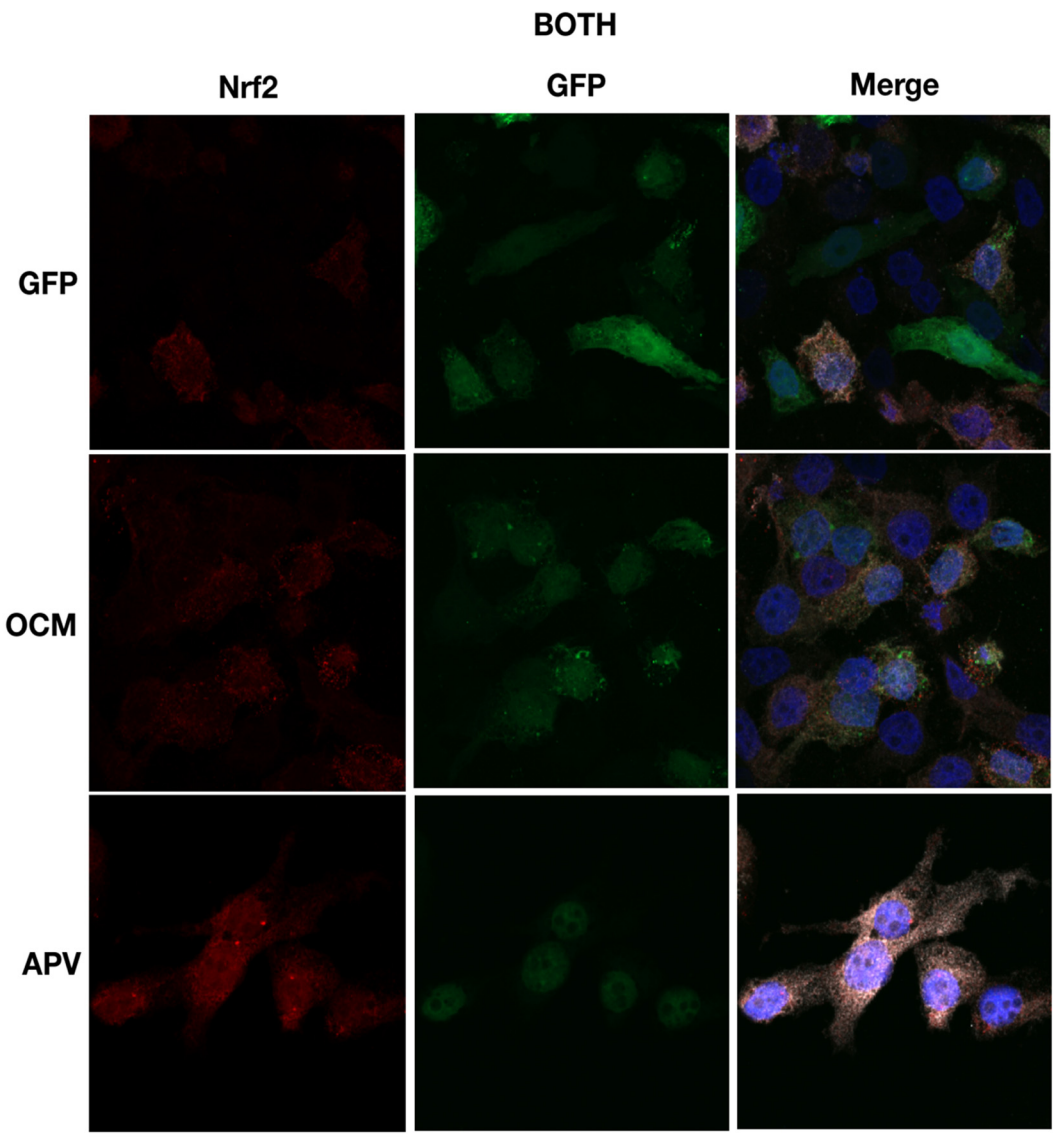

Figure 7. Cells treated with both AMA and Tg had the lowest transfection rate.

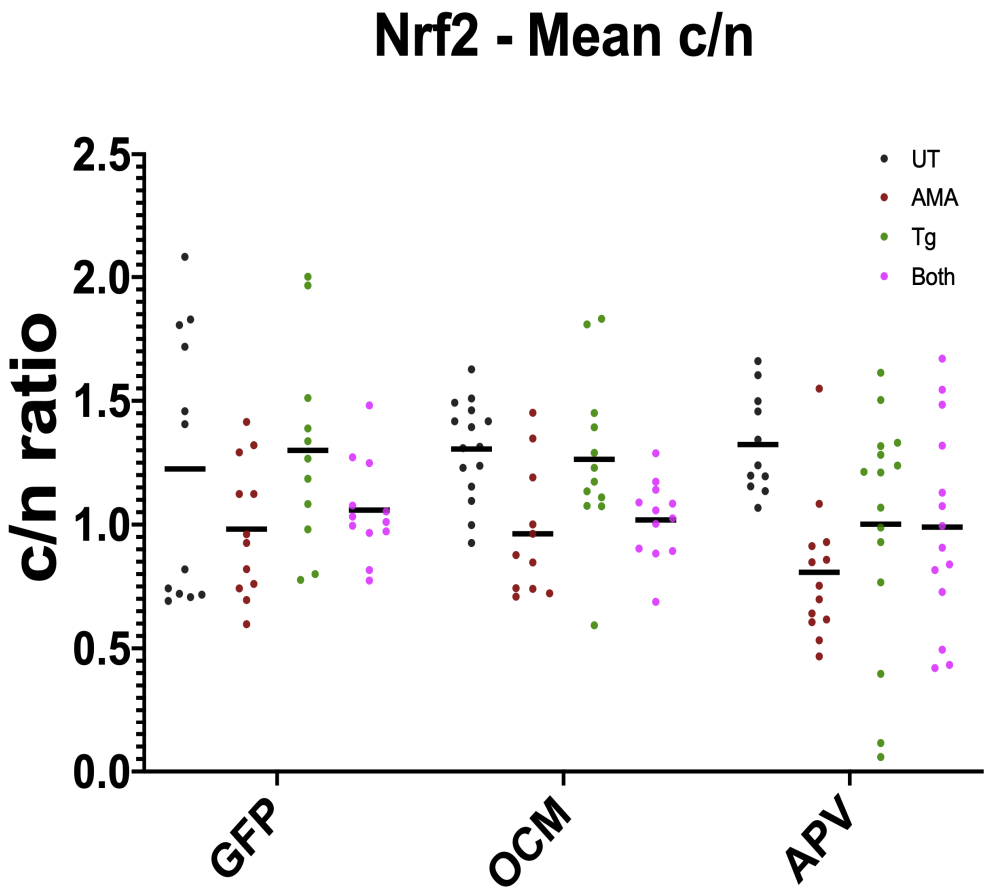

Figure 8. When the confocal images were analyzed through FIJI and the c/n ratios were compared, we observed that the presence of OCM does not significantly translocate the Nrf2 protein into the nucleus compared to GFP transfected cells. It was also observed that treatment with AMA and the combination of AMA and Tg(Both) resulted in more Nrf2 translocation to the nucleus within the cell type of GFP and OCM. This was not true for APV. In general, it seemed like APV transfected cells had more Nrf2 translocation when treated with AMA, Tg and Both. We observed a wider variation of c/n ratios for APV compared to GFP and OCM.

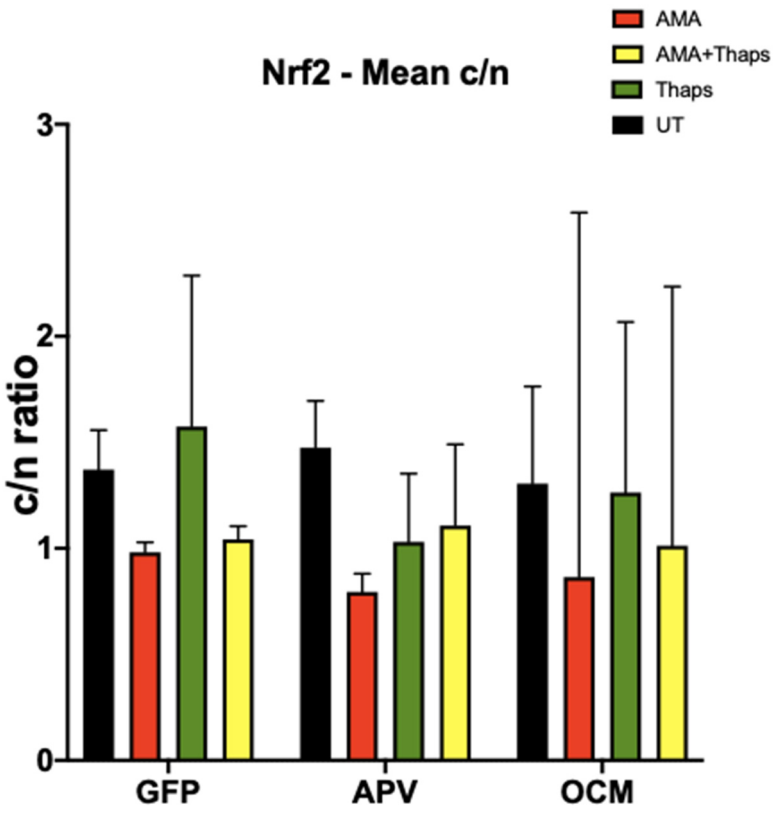

Figure 9. We analyzed the c/n ratios using two way ANOVA, using GFP as our control. Based on the analysis, we found no statistical significance.

CHAPTER FOUR

Discussion

Based on the LD50 experiments, we discovered that HeLa cells need higher levels of AMA and Tg dosages compared to HEK cells in order to reach 50% death. Based on the graph slope of LD50 graphs, the HeLa cells were more abruptly affected by cytotoxicity and experienced a more immediate 50% cell death compared to the HEK cells treated with AMA, as shown by its slope which was more linear. In order to gain a more accurate insight into the LD_{50} measurements for the HeLa cells, we can drug the cells with narrower differences of dosages, which will allow us to find the narrower range of LD_{50} dosage. In general, it can be concluded that HEK cells are more sensitive and less resistant against AMA and Tg compared to the HeLa cells.

When we carried out the MTS assays, HEK cells reacted according to the LD50 results, experiencing around 60-70 percent cell death with the calculated dosages. Based on the MTS assay graph, although there was an increase in cell survival rate in the presence of OCM, it was not significant. On the other hand, HeLa cells did not respond to the LD50 dosages of AMA and Tg as expected. This might be because of the inaccuracies that arose from the protocol and timeline of transfection experiments. This shows that AMA dosage needs to be higher in HeLa transfection experiments. We repeated the dosage response curve to come up with new concentration that will result in cell death closer to 50% of cell culture. The new dose was 72.20 uM.

TOM 20 is a marker for mitochondria and Bax is a marker for apoptosis. The untransfected cells were treated with AMA and Tg and then immunostained with TOM20

and Bax. Based on qualitative analysis, the untreated HeLa cells had the highest cell survival rate and healthier nucleus compared to the cells treated with AMA and TG. Although we do not know the reason behind why TOM20 is decreasing in expression compared to the untreated cells, we can conclude that with mitochondrial stress, there is less TOM20 expression in our confocal images. We can confirm this in future experiments by quantifying confocal images or using TOM 20 antibodies in western blots.

In the presence of mitochondrial stressors, there was a certain phenotype that reflected a change in morphology. With the untreated HeLa cells, due to the absence of mitochondrial stressors, the mitochondria looked more elongated and expanded, but in the presence of mitochondrial stressors, the mitochondria looked smaller and circular. In a similar manner, the nucleus in the control looked bigger in size and more circular compared to the AMA and Tg treated cells, which implies that AMA and Tg altered mitochondria and nuclear health which could account for the cytotoxicity observed in the MTS assay. Also, based on the images, we observed that Bax colocalizes with TOM20 which indicates the apoptosis process (Suen et al. 2008).

We took the images of HeLa cells and measured the fluorescence of the nucleus and the cytoplasmic to calculate the c/n ratio. Lower ratio shows the increase of translocation of Nrf2 proteins from the cytoplasm into the nucleus. In order to see whether OCM functions as a buffer for mitochondrial stress, the OCM transfected cells were compared with GFP and APV transfected cells. AMA and the combination between AMA and Tg resulted in lower instances of translocation compared to untreated and Tg

treated cells. The reason why treatment with 24 hours of Tg did not result in cell translocation of Nrf2 is possibly because prolonged treatment of Tg leads to apoptosis but shorter incubation periods can result in reversible mitochondrial fragmentation (Hom et al. 2007). This might mean that we looked at the wrong time point for Nrf2 translocation. Based on previous studies, prolonged Tg stimulation can induce apoptosis due to the Ca^{2+} depletion in the ER caused by ER stress (Jiang et al. 1994; Kaneko and Tsukamoto 1994). However, for apoptosis to happen, sufficient time of incubation is necessary. Based on previous experiments, it has been discovered that 48-hours of Tg incubation led to less than 40% of the initial survival with various ranges of dosages, indicating that cell death was happening (Hom et al. 2007). We think that our 24-hour incubation period of Tg was not enough time to induce the second phase of mitochondrial fragmentation in the HeLa cells. Based on this information, we can speculate that the cells only underwent the initial reversible phase of transient change, as tubular mitochondrial morphology was restored as a response to the decrease of Ca^{2+} levels within the cells and have not undergone the second phase of mitochondrial fragmentation, which irreversibly leads to cell death. Based on our Nrf2 data, we see that there is no significant difference between the c/n ratios of GFP and OCM transfected cells, which suggests that OCM and APV do not alter the translocation of Nrf2 after 24 hours of AMA and Tg treatment (**Figure 9**). However, we saw a statistical significance between untreated and AMA treated APV transfected cells. We also saw a statistical significance between untreated and AMA treated OCM transfected cells and between the untreated and the combination treatment among OCM transfected cells. Although we know that OCM functions as the calcium

buffer in mammalian cells, there could be many unidentified cellular activities that are affecting its function. In the future, we plan to further investigate the unique role of OCM in our cell culture models by treating the cells with AMA and Tg on different incubation time periods (0.5 hours, 3 hours, 6 hours, 24 hours, 48 hours), using other apoptotic markers such as cytochrome C, and taking higher resolution confocal images.

Abbreviations

GFP	Green Fluorescent Protein
APV	**α**-parvalbumin
OCM	Oncomodulin
CaBPs	Calcium Binding Proteins
AMA	Antimycin A
Tg	Thapsigargin
ROS	Reactive Oxygen Species
IHCs	Inner Hair Cells
OHCs	Outer Hair Cells
ER	Endoplasmic Reticulum

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