REPORT

ISN-CAEN Award

CATEGORY 1A: Visit by the applicant to another laboratory

Applicant: Wilasinee Suwanjang, Ph.D.

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Host: Professor Samuel H.H. Chan

Supervisor: Associate Professor Kay Wu

Host Laboratory: Institute of Translational Research in Biomedicine,

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Duration of visit: June 1, 2016 – August 24, 2016

Background of project

Life stress is considered to be a factor in development of psychiatric illnesses that induce impair it ability to appropriately regulate physiological and behavioral responses. Increasing evidence support the idea that chronic stress is a risk factor for the pathogenesis underlying neurodegeneration such as cognitive impairment or dementia. Stressor exposure activates the hypothalamic-pituitary-adrenal (HPA) axis and causes an elevated level of glucocorticoids (GC) from the adrenal glands. Our previous study found that cellular treatment with the toxic-high doses of synthetic GC receptor agonist, dexamethasone, showed a marked increase in the oxidative stress and cell death in neurons. In addition, prolonged exposure to stress induces neuropathological alteration such as dendritic atrophy. The exact mechanisms by which stress, especially chronic stress contribute to the damage of hippocampal neuron remains unclear. Therefore, the present proposed research is the first study to investigate the effect of stress-induced mitochondrial and signaling molecule alteration in neuronal model. This result may lead to gain more knowledge and development of a new therapeutic strategy for the treatment of depression-related neurodegeneration and dementia.

Objectives

The purpose of this study aim to investigate whether the effects on stress-induced neurodegeneration through the modulation of mitochondria damage and signaling molecule alteration. A novel action of mitochondrial may be important for future target treatment in depression-related neurodegenerative diseases and dementia.

1) To determine whether stress-induced mitochondrial dynamic impairment and neurodegeneration (in term of mitochondrial fission-fusion processes and neuron cell death).

2) To determine whether stress-induced neurodegeneration through ion alteration in neuronal cells.

Plan of work

1) To determine whether stress suppressed neuronal cell proliferation, the WST-1 assay was used to determine the assessment of cell proliferation after treatment of culture SH-SY5Y cells with dexamethasone.

2) To determine whether stress-related with the ATP concentration, ATP content was determined by ATP colorimetric assay kit.

3) For increasing oxidative stress in mitochondria, the immunofluorescent staining was used to determine level of superoxide in SH-SY5Y cells.

4) To determine whether stress-induced mitochondrial dynamic impairment, the mRNA and protein levels of mitochondria were measured by western blot and real-time quantitative RT-PCR.

5) To determine whether mitochondria movement in SH-SY5Y cells under dexamethasone condition. Mitochondrial dynamics were imaged with time-lapse microscope.

Results

In order to investigate the optimal concentration and incubation period of dexamethasone-induced toxicity, SH-SY5Y cells were treated with various concentrations of dexamethasone at 1, 2, 5, 10 and 20 µM for 6 and 24 hr, respectively. Cell proliferation was measured using WST-1 assay, based on the enzymatic cleave of the tetrazolium salt WST-1 to a yellow-orange soluble formazan product by mitochondria dehydrogenase, which are a part of the mitochondrial respiration chain. Cellular damage precludes cells from generating the enzyme required for metabolic function and growth. Dexamethasone at 1, 2, 5, 10 and 20 µM for 6 and 24 hr significantly decreased cell proliferation compared with untreated (0 µM dexamethasone) control values, respectively (Figure 1).

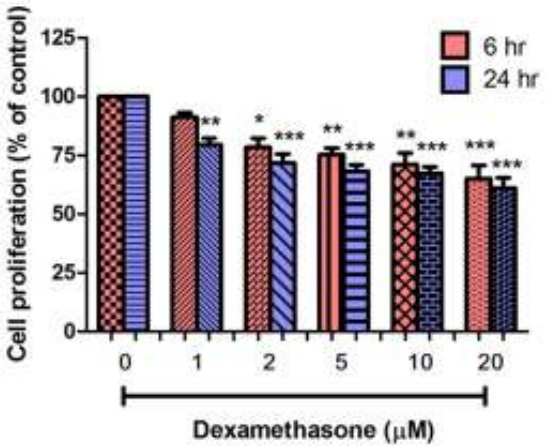

Figure 1: The effect of dexamethasone-induced reduction in cell proliferation. SH-SY5Y cells were treated with various concentrations of dexamethasone for 6 and 24 hr. The control-cultured cells (0 µM dexamethasone) were incubated with culture medium for 6 and 24 hr. Cell proliferation was measured using WST-1 assay. The results are expressed as mean ± S.E.M. of four independent experiments. One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons test was performed for statistical analysis. *P<0.05, **P<0.01 and ***P<0.001 compared with control.

Caspase-3 is one of the cysteine protease activities implicates in apoptosis. In the presence of cleaved caspase-3 which is the active form that from the proteolytic activation of pro-caspase-3. Because pro-caspase-3 (35 kDa) is cleaved into 19 or 17 kDa subunit. Treated

cells with 10 and 20 µM dexamethasone for 6 and 24 hr significantly increased the activated form of caspase-3 (19 kDa and 17 kDa) when compared with untreated cells. Therefore, these results show that dexamethasone induced caspase-3 activation in a dose dependent manner (Figure 2).

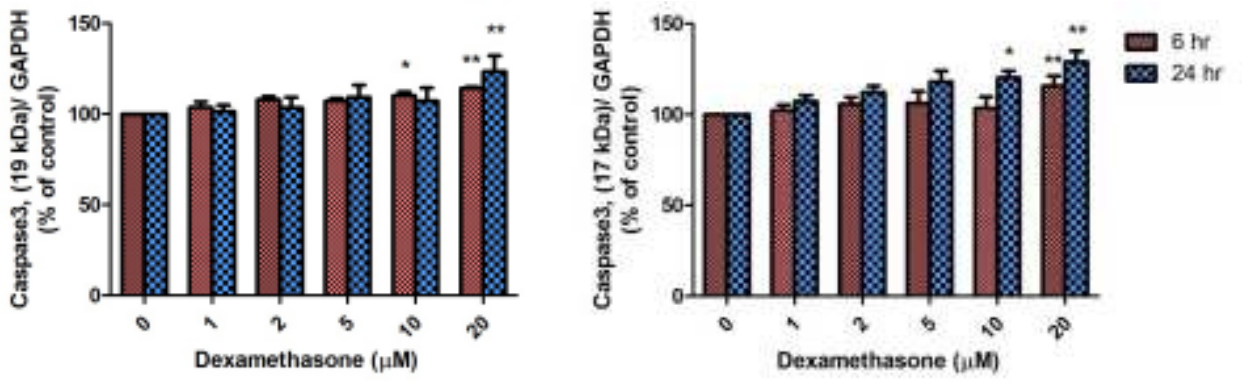

Figure 2: Effect of dexamethasone treatment on caspase-3 and cleaved caspase-3 level. SH-SY5Y cells were treated with 1, 2, 5, 10 and 20 µM dexamethasone for 6 and 24 hr. The levels of caspase-3 and cleaved caspase-3 were determined using Western blot analysis. Protein bands for each regimen were quantified by densitometry and their differences are represented in graph as a ratio of caspase-3 (35 kDa) and cleaved caspase-3 (19 and 17 kDa) over GAPDH bands. Values represent mean ± S.E.M. of four separate determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with control-untreated cells.

To detect intracellular ATP content, SH-SY5Y cells were incubated with various concentration of dexamethasone (1, 2, 5, 10 and 20 µM) for 6 and 24 hr and ATP content was measured by ATP colorimetric assay kit. The decrease in intracellular ATP in the presence of dexamethasone was statistically significant at 5 µM for 6 hr and 1, 2, 5 and 20 µM for 24 hr (Figure 3).

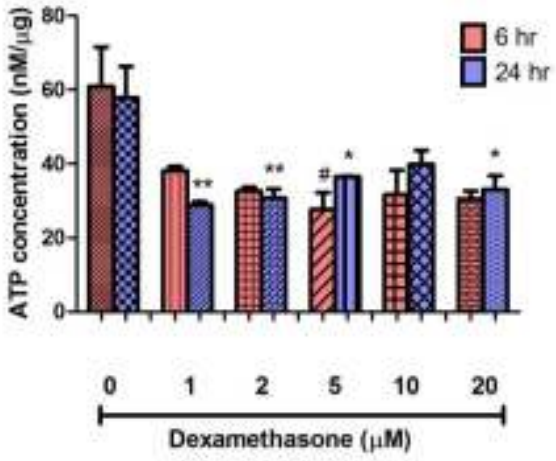

Figure 3: Effect of dexamethasone on intracellular ATP content in SH-SY5Y cells. Cells were treated with various concentration of dexamethasone for 6 and 24 hr. Intracellular ATP content was determined using ATP colorimetric assay kit. Values represent mean ± S.E.M. of three separate determinations. *P<0.05 and **P<0.01 compared with control (0 µM).

Mitochondrial are morphologically dynamic organelles that continuously divide and fuse to form small individual units or interconnected network within the cells (Bereiter-Hahn, 1990). The equilibrium between two states in healthy cells is regulated by the relative roles of fission and fusion proteins. The effect of dexamethasone-induced alteration in fission proteins (DRP1, pDRP1 and FIS1) and fusion (OPA1 and MFN2) were determined in SH-SY5Y cells using Western blot analysis. SH-SY5Y cells were treated with various concentration of dexamethasone for 6 and 24 hr. The control cells were incubated in cultured medium for 6 and 24 hr. DRP1 is an essential mediator of apoptosis-induced mitochondria fission. It translocases from the cytoplasm to mitochondria and form cluster at mitochondrial outer membrane whereas phosphorylated DRP1 has been identified as a post-translational modification to regulate DRP1 medicated mitochondrial fission. Dexamethasone at 2, 5, 10 and 20 µM for 6 hr significantly decreased the amount of pDRP1 expression when compared with control. In addition, dexamethasone at 10 and 20 µM for 24 hr significantly decreased the amount of pDRP1 expression. Fission protein DRP1 slightly decreased and significantly decreased after 20 µM dexamethasone-treated cells for 24 hr, respectively (Figure 4). Another mitochondria fission protein, FIS1, dexamethasone at 10 and 20 µM for 24 hr significantly decreased in the FIS1 protein level (Figure 5). This agrees with our previous report that dexamethasone treatment significantly increase fusion proteins expression

(Suwanjang et al., 2016). Of note, however, reduction of FIS1 expression resulting mitochondrial dynamic changes, including a reduction of fission and mitochondrial elongation (Lee et al., 2007). Together, these data suggest that mitochondrial fission is suppresed in response to dexamethasone and that association of DRP1 with mitochondria might be regulated by ATP content in the neuronal cells.

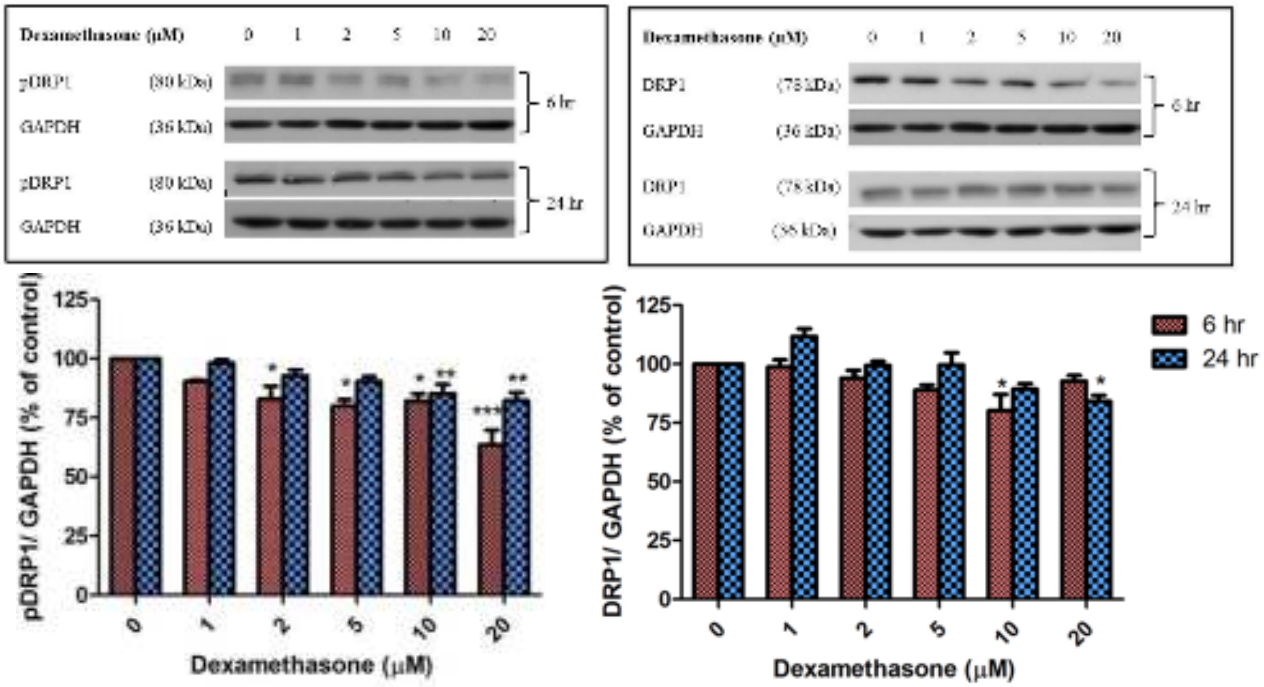

Figure 4: Effect of dexamethasone-induced alteration in pDRP1 and DRP1 proteins levels in SH-SY5Y cells. Cells were treated with various concentration of dexamethasone for 6 and 24 hr. The levels of pDRP1 and DRP1 were determined using Western blot analysis. Protein bands for each regimen were quantified by densitometry and their differences are represented in graph as a ratio of pDRP1 or DRP1 over GAPDH bands. Values represent mean ± S.E.M. of four separate determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with control-untreated cells.

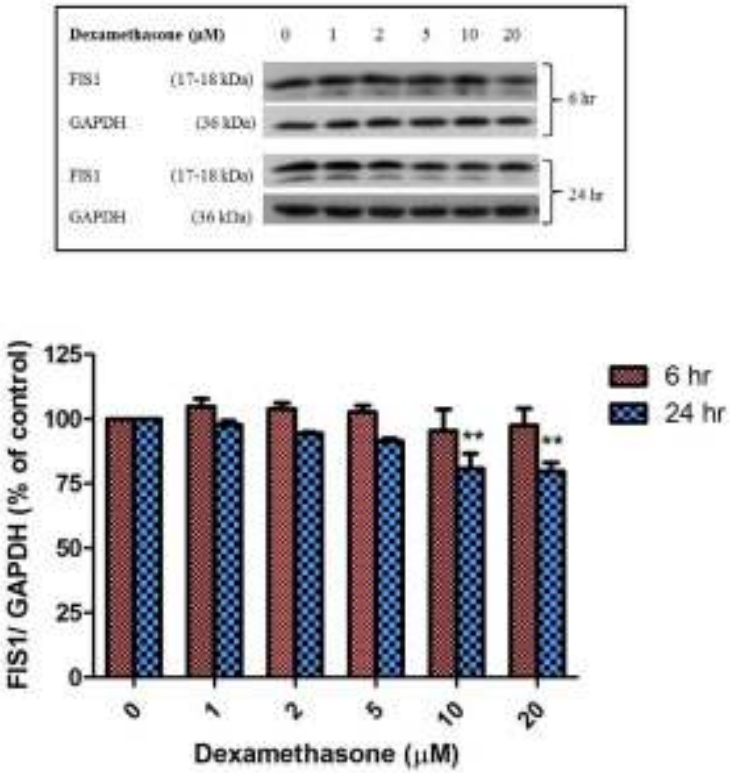

Figure 5: Effect of dexamethasone-induced alteration in FIS1 protein levels in SH-SY5Y cells. Cells were treated with various concentration of dexamethasone for 6 and 24 hr. The levels of FIS1 were determined using Western blot analysis. Protein bands for each regimen were quantified by densitometry and their differences are represented in graph as a ratio of FIS1 over GAPDH bands. Values represent mean ± S.E.M. of four separate determinations. ***P*<0.01 compared with control-untreated cells.

To test wherther dexamethasone induce alteration of mitochondrial fusion protein expression in SH-SY5Y cells. The large GTPases, OPA1and MFN2 play a role in fusion processes of mitochondrial dynamics. OPA1 is localized on the inner mitochondrial membrane and required for inner mitochondrial membrane fusion. The various variants of OPA1 are diffentially proteolyzed into long and short forms. The cleaved forms of OPA1 are more loosely attached to the inner mitochondrial membrane than long forms that retain a hydrophobic domain (Ishihara et al., 2006). Whereas MFN1 and MFN2 are located into the outer mitochondrial membrane (OMM). MFN2 trigger aggregation of mitochondrial into tight networks through close interaction between OMM, followed by outer membrane fusion that result in formation of

enlarged mitochondria. Dexamethasone at 5, 10 and 20 μM for 24 hr significantly increased the amount of OPA1 (92 kDa) (Figure 6), while dexamethasone at 10 and 20 μM for 24 hr significantly increased the amount of MFN2 when compared with control values (Figure 7), respectively. However, dexamethasone treated for 6 hr was not significantly changes in overall mitochondrial fusion protein (both OPA1 and MFN2). These data suggest that dexamethasone exposure for 24 hr-induced mitochondria fusion is caused by induction of OPA1 and MFN2 protein levels. Supporting the alteration of MFN2 and OPA1 expression-mediated the ubiquitin proteosome pathway in dexamethasone-treated SH-SY5Y cells.

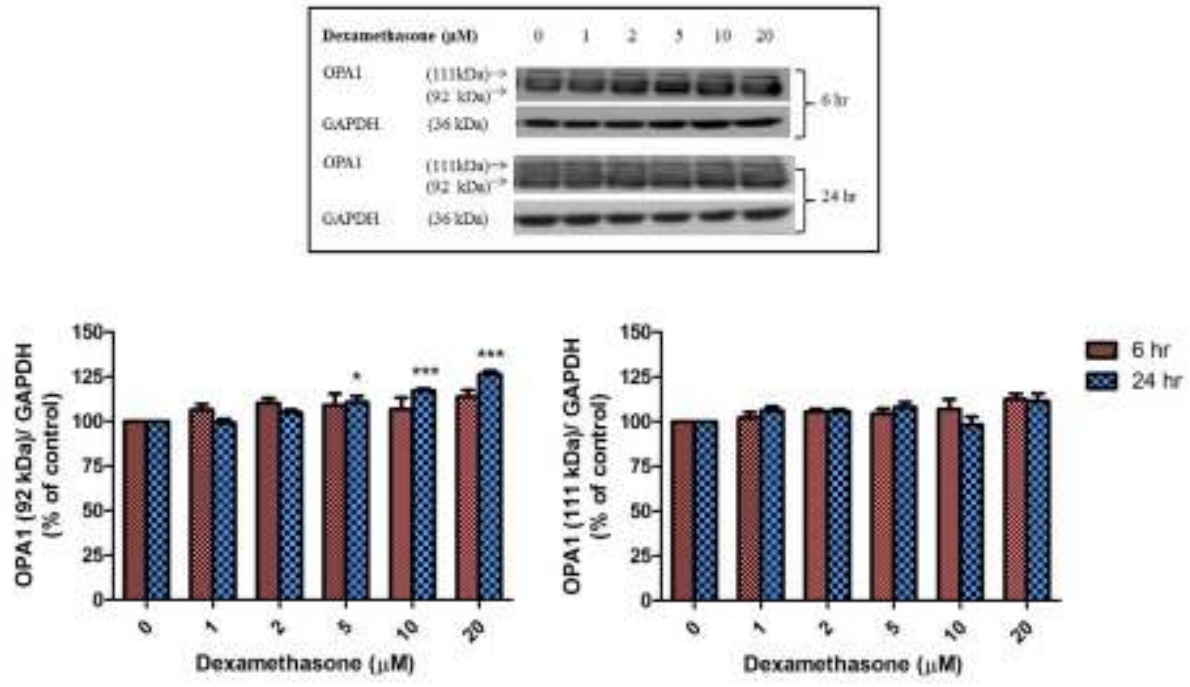

Figure 6: Effect of dexamethasone-induced alteration in OPA1 proteins levels in SH-SY5Y cells. Cells were treated with various concentration of dexamethasone for 6 and 24 hr. The levels of OPA1 were determined using Western blot analysis. Protein bands for each regimen were quantified by densitometry and their differences are represented in graph as a ratio of pDRP1 or DRP1 over GAPDH bands. Values represent mean ± S.E.M. of four separate determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with control-untreated cells.

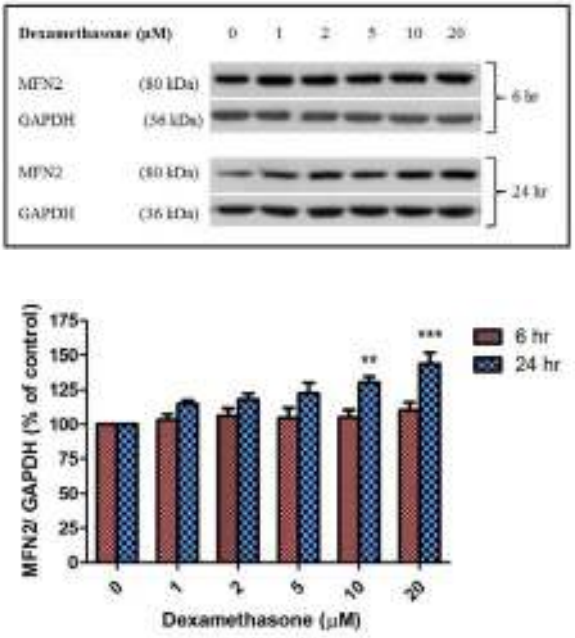

Figure 7: Effect of dexamethasone-induced alteration in MFN2 protein levels in SH-SY5Y cells. Cells were treated with various concentration of dexamethasone for 6 and 24 hr. The level of MFN2 was determined using Western blot analysis. Protein bands for each regimen were quantified by densitometry and their differences are represented in graph as a ratio of MFN2 over GAPDH bands. Values represent mean ± S.E.M. of four separate determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with control-untreated cells.

To determine whether dexamethasone altered mitochondrial movement, the confocal cell^{R} time-lapse imaging on 20 μM dexamethasone-treated SH-SY5Y cells with mitotracker staining. Mitochondrion staining appears as green color. These data raised the possibility that dexamethasone disruption of mitochondria quality control and cause the accumulation of mitochondrial fusion in neuronal SH-SY5Y cells (Figure 8). In additon, the damaged of neuronal cells, mitochondria need to be shuttled back to the cell body for turnover by mitophagy.

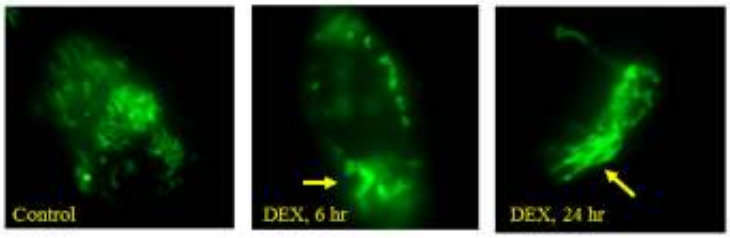

Figure 8: Mitochondrial morphology effect of dexamethasone-treated neuronal SH-SY5Y cells. Cell^{R} image of

The data presented in the previous section raise the possibility that dexamethasone at high concentration caused mitochondrial dynamics alteration and ATP content reduction in SH-SY5Y cells. The mitochondria quality control is achieved through fission and fusion processes. Damaged mitochondria can fuse with healthy mitochondria to restore the level of healthy components necessary for proper mitochondrial function through mitophagy process. The components of mitochondrial quality control such as PINK1 and PARKIN.

To assess whether PINK1 expression correlate with ATP content under high dose dexamethasone-treated SH-SY5Y cells using Western blot analysis. SH-SY5Y cells were treated with various concentration of dexamethasone (1, 2, 5, 10 and 20 μM) for 6 and 24 hr. The control cells were incubated in cultured medium only for 6 and 24 hr. Dexamethasone at 5, 10 and 20 μM for 24 hr significantly decreased the amount of PINK1 when compared with the control values, respectively. Whereas dexamethasone at 20 μM for 6 and 24 hr significantly increased in the PARKIN protein level when compared with the control values (Figure 8). The result indicate that PINK1 and PARKIN expression levels exhibit its response to the level of ATP content under the high dose of dexamethasone treatment in SH-SY5Y cells.

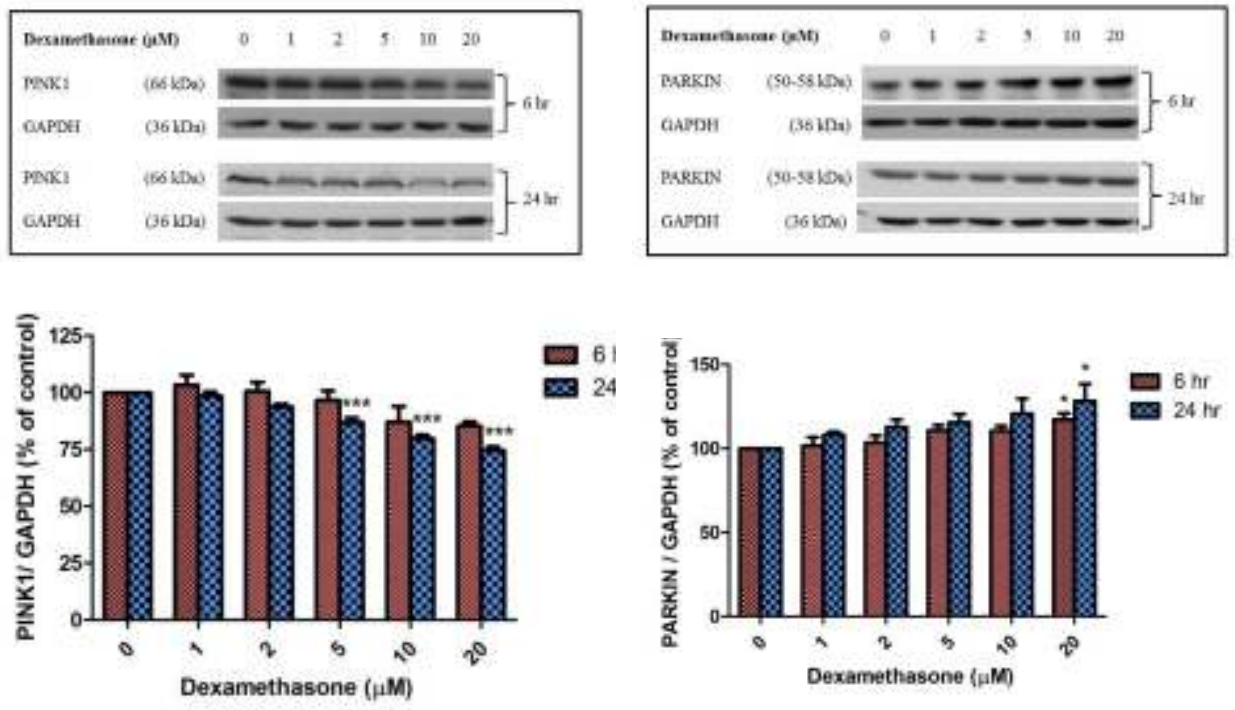

Figure 8: Effect of dexamethasone-induced alteration in PINK1 and PARKIN protein levels in SH-SY5Y cells. Cells were treated with various concentration of dexamethasone for 6 and 24 hr. The levels of PINK1 and PARKIN were determined using Western blot analysis. Protein bands for each regimen were quantified by densitometry and their differences are represented in graph as a ratio of PINK1 and PARKIN over GAPDH bands. Values represent mean ± S.E.M. of four separate determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with control-untreated cells.

Dexamethasone-induced cytosolic ROS production was first detected with dihydroethidine (HEt) fluorescence, which is a fluorogenic indicator of ROS (Suwanjang W. et al., 2016). Next, the present study investigated the effect of dexamethasone on ROS production in mitochondria. The SH-SY5Y cells were treated with various concentration (0-20 μM) of dexamethasone for 24 hr. The cells were incubated with MitoTracker Green for 1 hr and MitoSOX^{TM} Red staining for 30 min. MitoSox-red is reported used to assay the presence of superoxide generated specifically from mitochondria (Kirkland *et al.*, 2007). The results revealed that the intensity of MitoSOX in by dexamethasone-treated cells was increased compared with the intensity of MitoSOX by 0 μM dexamethasone-treated cells (native control) (Figure 9).

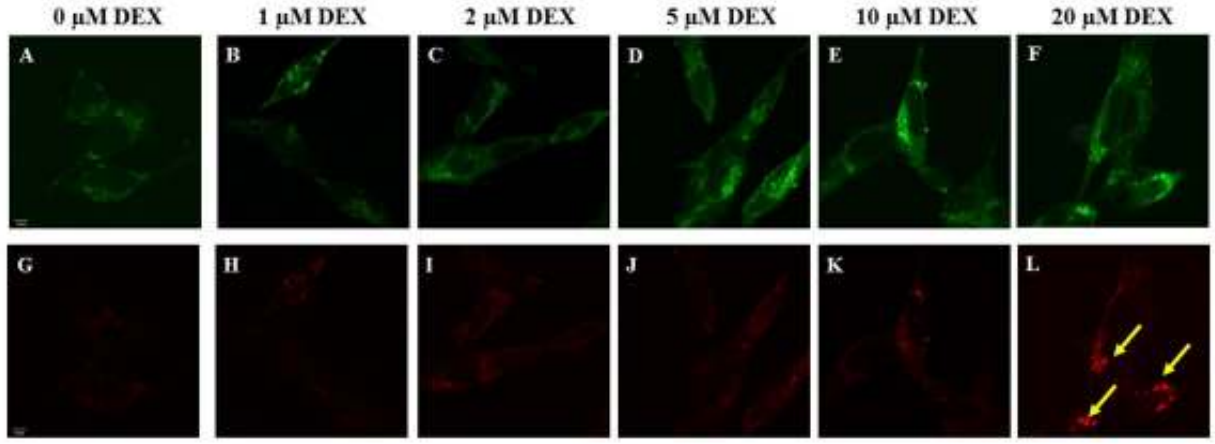

Figure 9: MitoSOX Red localizes to mitochondria of dexamethasone treated cells. Cells loaded with MitoSOX Red and MitoTracker Green for 30 min and analsed by an array confocal laser scanning microscope.

We can conclude that cellular treatment with the synthetic GCs, dexamethasone injur mitochondria by decreasing the level of ATP, mitochondrial fission protein. Dexamethasone-induced toxicity also caused an increase in mitochondria ROS production, mitochondrial fusion proteins, and the activation of the PINK1-PARKIN, along with an increase in caspase-3 activation. The dexamethasone-treated cells can suppress proliferation in neuronal cells.

References

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Suwanjang W., Abramov AY., Charngkaew K., Govitrapong P., Chetsawang B. Melatonin prevents cytosolic calcium overload, mitochondrial damage and cell death due to toxically high doses of dexamethasone-induced oxidative stress in human neuroblastoma SH-SY5Y cells. Neurochem Int. 2016;97:34-41.

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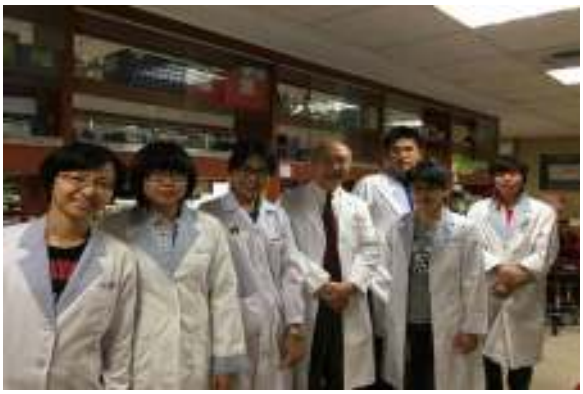

With Professor Samuel H.H. Chan, Professor Julie Y.H. Chan and lab member.

Farewell dinner with the member of Institute of Translational Research in Biomedicine

With Assoc.Prof. Kay L.H. Wu and lovely friends at Tainan and Taichung