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# Neuroprotection during Hypoxia using Steroids Analogues

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Hypoxic-ischemic (HI) brain injury is one of the main causes of long-term neurologic disability, morbidity and, death worldwide in adults and children. This public health concern is mainly characterized by a decrease in oxygen concentration and blood flow to the tissues, which leads to cell death by energy depletion and increases free radical generation and inflammation, caused by an inefficient supply of nutrients to the brain. Hypoxic-ischemic brain injury occurs in perinatal asphyxia (PA) [1, 2]. This clinical condition is characterized by oxygen supply suspension before, during, or immediately after birth and it is an important risk factor for neurodevelopmental damage. Its estimated 1/1000 live births incidence in developed countries rises from FIVE to TENfold in developing countries. Neuroactive steroids like selective estrogen receptor modulators, SERMs, exert several neuroprotective effects in different pathological scenarios. These include a decrease in reactive oxygen species, mitochondrial survival, and maintenance of cell viability. In this context, these neurosteroids constitute promising molecules, which could modify brain response to injury. To replicate the conditions that occur in the presence of deprivation or decrease in O2 and glucose levels several studies have proposed a model of O2 and glucose deprivation (OGD) [2]. For this study T98G cells were seeded onto 24-well plates in DMEM culture medium containing 10% FBS at a seeding density of 10,000 cells per well and incubated for 2–3 days until they reach confluence. Subsequently, the medium was changed to glucose-free DMEM, and then incubated in 1% O2 in a hypoxia incubator (Hypoxia Incubator Chamber, STEMCELL) for 9 hours. This was followed by reperfusion by changing the media to high glucose DMEM supplemented with 10% FBS and transferring the cells to 37°C in 95% air/5% CO2 in normal conditions, the control group was maintained in normal conditions of O2 and glucose during the experiment. For drug treatments, cell cultures were incubated in DMEM serum-free medium containing 10 and 100 nM Raloxifene and Tibolone, as co-treatment of OGD and reperfusion. Cell viability and morphology are tightly related, and increases in oxidative stress, lipid peroxidation, and changes in the mitochondrial membrane potential usually induce noticeable morphological changes. Determination of Mitochondrial Mass and mitochondrial membrane potential was evaluated using Nonyl acridine orange (NAO) and Tetramethyl Rhodamine Methyl Ester (TMRM) respectively by flow cytometry and fluorescence intensity. After treatments were completed, cells were loaded in the dark with 5 µM NAO at 37°C for 20 min. Thereafter, cells were washed with PBS to eliminate all non-sequestered dye. DIC and fluorescence images were obtained with NIKON - Eclipse Ti-E PFS microscope. The analysis of cellular fluorescence was performed using Fiji. The microphotographs were loaded, and background signals were eliminated from the images. Subsequently, 50 cells were randomly selected using a numbered grid in each microphotograph. The mean fluorescence value of the 50 cells was determined in eight microphotographs for each treatment using the Measure algorithm of Fiji and selecting each cell manually via ROI's (Regions of Interest) Management. There were no variations in the conditions of the image processing. Each assay was performed with a minimum of six replicate wells for each condition and experiments were repeated three times. Qualitative results suggested that OGD may induce morphological changes, cells showed smaller cell bodies and fewer cellular processes than control cells. Raloxifene and tibolone preserved cell morphology even in cells exposed to OGD. Assays showed a significant viability reduction on cells exposed to OGD, cells co-treated with 100 and 10 nM raloxifene and 100 nM tibolone showed a 65,34 % (p= 0,0021), 70,56 % (p <0,0001) and 66,49 % (p= 0,0013) increase in cell viability when compared to OGD cells, respectively. Regarding lipid peroxidation, the assay showed a significant preservation of mitochondrial mass using a cotreatment of Raloxifene 100 nM (p= 0,0178) and 10 nM (p=0.0014). On the other hand, Tibolone 10 nM (p= 0,0137) cotreatment preserves significantly mitochondrial membrane potential in cells exposed to OGD/reperfusion. Our results suggest that raloxifene and tibolone exert protective effects in human T98G glial cells exposed to OGD/reperfusion through the reduction of lipid peroxidation, the preservation of mitochondrial membrane potential and viability, which altogether counteract the cell damage in our model.

## References

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