HYPOXIA AFFECTS THE EXPRESSION OF SOX GENES AND INDUCTION OF NEURAL DIFFERENTIATION OF HUMAN EMBRYONAL CARCINOMA NT2/D1 CELLS

Stefan Lazic[1], Filip Dužanic[1], Danijela Stanisavljevic Ninkovic[1], Danijela Drakulic[1], Marija Mojsin[1], Milena Milivojevic[1],Vanda Balint[1],, Isidora Petrovic[1],Natasa Kovacevic Grujicic[1], Marija Schwirtlich[1] and Milena Stevanovic[1, 2, 3]

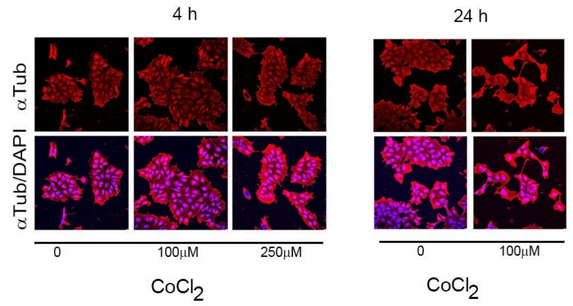
[1]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia
[2] University of Belgrade, Faculty of Biology, Belgrade, Serbia
[3]Serbia Serbian Academy of Sciences and Arts, Belgrade, Serbia

INTRODUCTION:

Adult neurogenesis is a complex process of differentiation of neural stem cells in neurons during adult life and it takes place in very limited regions of the central nervous system - neurogenic zones. The roles of adult neurogenesis are the maintenance of brain homeostasis, brain plasticity, memory formation and learning. Following injury, a reduced supply of glucose and oxygen in damaged areas cause death to a large number of cells. The capacity of adult neurogenesis does not have sufficient potential to completely restore a neuronal network and function. SOX transcription factors govern diverse cellular processes during embryonic and adult neurogenesis, such as maintaining the multipotency of neural stem cells, cell proliferation, cell fate decision as well as terminal differentiation of neurons and glia. Impaired miRNAs profiles which were detected following cerebral ischemic stroke provided evidence that modulation of their expression could be considered as diagnostic and prognostic tool providing basis for potential therapeutic strategy. Aim of this study was to investigate the expression of *SOX* genes and miRNAs in cells following ischemia. Neuronal differentiation of human pluripotent embryonal carcinoma stem cell line NT2 / D1 was used as an *in vitro* model system for studying the process of human neurogenesis. Chemical hypoxia was induced with cobalt chloride ($CoCl_2$).

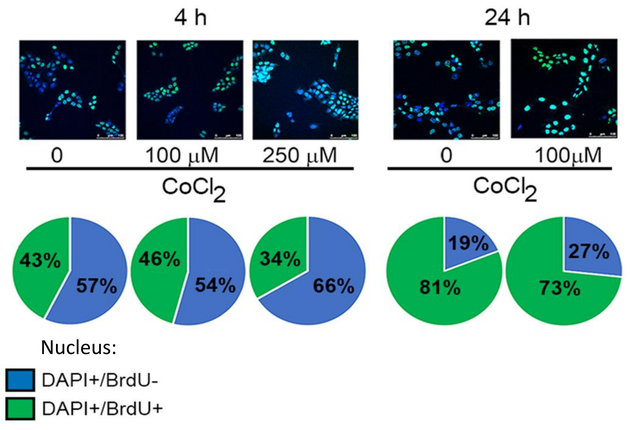
RESULTS:

1. Analysis of the effect of hypoxia on NT2/D1 cell morphology



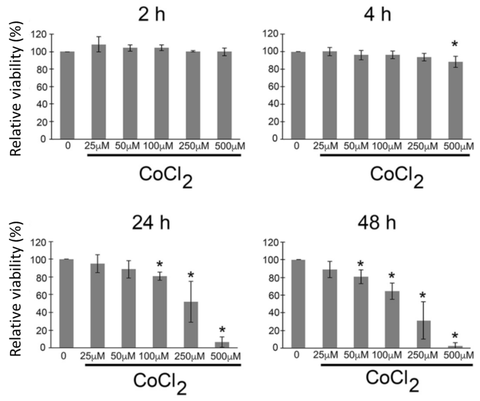
Immunocytochemical analysis of α-Tubulin expression (red) in cells treated for 4 and 24 hours. The cells were analyzed on a confocal microscope. αTUB - α-tubulin; DAPI - 4', 6'-diamidino-2'-phenylindole.

2. Analysis of the effect of hypoxia on NT2 / D1 cell proliferation



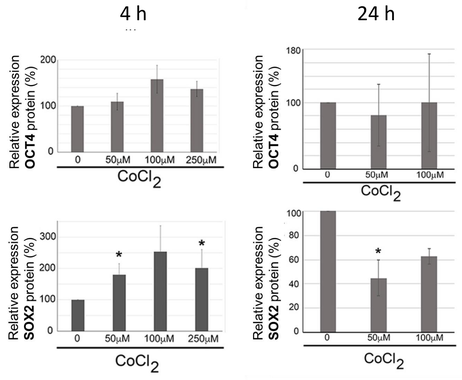
Immunocytochemical detection of BrdU (green) in the nucleus of NT2 / D1 cells after 4 and 24 hours treatment in the presence of cobalt chloride. The cell nuclei were stained with DAPI (blue). Graphic view percentage of BrdU positive nuclei in NT2 / D1 cells. The percentage was calculated on a minimum 200 counted DAPI positive nucleus per treatment. DAPI - 4 ', 6'-diamidino-2'-phenylindole.

3. Analysis of the effect of cobalt chloride treatment on the viability of NT2 / D1 cells



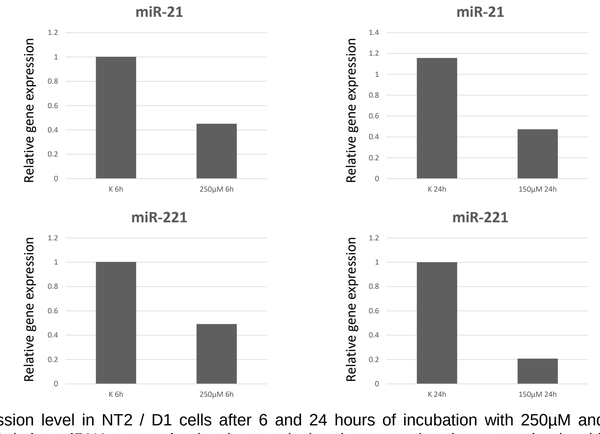
Histograms represent a statistical analysis of the relative viaibility of NT2 / D1 cells after treatment with different concentration of cobalt chloride (0.25 μM, 50 μM, 100 μM, 250 μM and 500 μM) for 2, 4, 24 and 48 hours. Cell viability measured by MTT assay and is expressed as the percentage of relative change in cell viability relative to control cells (0) assigned a value 100.

4. Analysis of the effect of hypoxia on the expression of proteins involved in the regulation of maintenance of pluripotent cell characteristics



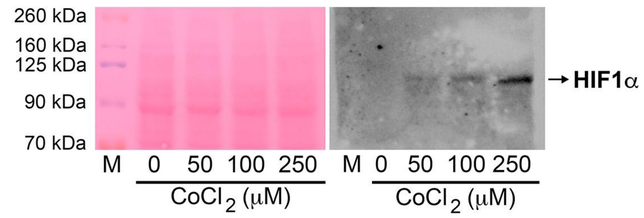
Markers of pluripotency, OCT3 / 4 and SOX2 proteins were analysed by Western blot in NT2 / D1 cells after 4 and 24 hours of incubation at different concentrations of cobalt chloride. Relative protein expression is calculated as a percentage of relative changes in expression relative to the control cells (0) to which it was assigned value 100. Expression of Actin was detected in order to control equally applied amounts of protein.

5. Analysis of the effect of hypoxia on the miRNK expression level of NT2 / D1 cells



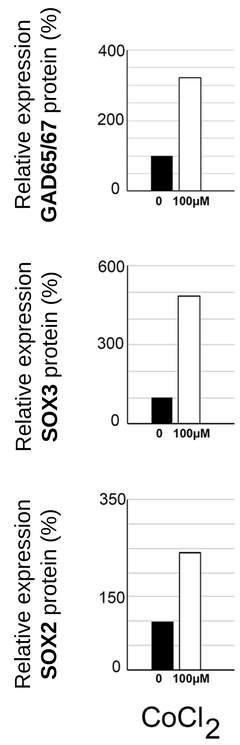
miRNA expression level in NT2 / D1 cells after 6 and 24 hours of incubation with 250μM and 150μM $CoCl_2$ respectively. Relative miRNA expression level was calculated compared to the expression level in control group (untreated NT2/D1 cells). Results were obtain by quantitative real time PCR method.

6. Analysis of the effect of selected concentrations of cobalt chloride on HIF1α protein expression in NT2 / D1 cells



Western blot analysis of Hypoxia inducible factor 1α (HIF1α) protein in untreated NT2 / D1 cells and in cells grown in the presence different concentrations of cobalt chloride for 4 hours.

7. Analysis of the effect of hypoxia on the induction of neural differentiation of NT2 / D1 cells



Histograms represent an analysis of the expression levels of GAD65 / 67, SOX3 and SOX2 proteins in cells induced for 7 days with retinoic acid. Relative protein expression is expressed as the percentage of relative change in expression in the cells relative to the control cells (0) to which it is assigned value 100. Results were obtained by Western blot method.

CONCLUSIONS:

- Stress caused by cobalt chloride treatment for 24 hours affected cell proliferation and cell morphology of NT2 / D1 cells.
- Treatment with cobalt chloride for 4 hours in concentrations of 50 μM, 100 μM and 250 μM led to an increase in HIF1α protein expression level in NT2 / D1 cells.
- Stress caused by treatment with cobalt chloride for 4 and 24 hours significantly affected SOX2 protein expression level in NT2 / D1 cells.
- Stress caused by treatment with cobalt chloride for 4 and 24 hours significantly affected OCT4 protein expression level in NT2 / D1 cells.
- Stress caused by treatment with cobalt chloride for 4 and 24 hours significantly affected miR-21 and miR-221 gene expression level in NT2 / D1 cells
- Stress caused by treatment with cobalt chloride for 24 hours affected the efficiency of induction of neuronal differentiation of NT2/D1 cells. Different level of GAD65/67, SOX2 and SOX3 protein expression, compared to control cells was detected in neural progenitors after 7 days treatment of cells with RA