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## The Effects of Hypoxia on Pattern and Pattern formation in cortical B50 Neuronal cells in culture

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**ABSTRACT:** Hypoxia has been implicated in nerve cell deaths that occur in a variety of neurological disorders and opioid receptor agonists have been shown to have some positive benefits on the nervous system. The aim of the present work was to investigate the effects of hypoxia and opioid receptor agonists' treatment on the morphology of B50 neuronal cell lines cultured in hypoxia using neuronal pattern and pattern formation as a case study. The B50 cells were cultured in normal incubator (21% O<sub>2</sub>; 5% CO<sub>2</sub>) as control group and hypoxic incubator (5% O<sub>2</sub>; 5% CO<sub>2</sub>) as the experimental group, three opioid receptor agonists namely DAMGO ( $\mu$ ) DSLET ( $\delta$ ) and ICI-199,441 ( $\kappa$ ) were administered to the cells for 48 hours as treatment against hypoxia after 48 hours of culture at dose of 10 $\mu$ M, 50 $\mu$ M and 100 $\mu$ M. Neuronal morphology and wellbeing was assessed using same field morphological assessment and lactate dehydrogenase leakage (LDH). The result showed groups of dead and degenerating B50 neuronal cells, altered neuronal pattern and pattern formation and some significant changes ( $P < 0.05$ ) in cellular levels of LDH leakage in normal B50 cells (100%), hypoxic cells (587%), and treated cells with 100 $\mu$ M DAMGO( $\mu$ ) (143%), 50 $\mu$ M DSLET( $\delta$ ) (140%) and 50 $\mu$ M ICI-199,441 ( $\kappa$ ) (109%). The changes in morphology, neuronal pattern and LDH release indicate that hypoxia induced morphological and cellular changes in B50 cells in hypoxia and that B50 neuronal cells are susceptible to damage and injurious effects of hypoxia as most brain cells while opioid agonists have some potential benefits in the treatment of hypoxia-induced changes in neuronal B50 cells in culture.

**Keywords:** Hypoxia, B50 Neuronal cells, neuronal morphology, neuronal pattern, opioid agonists, neurodegeneration

### Introduction

Hypoxia can lead to oxidative stress which has been implicated in nerve cell death that occurs in a variety of neurodegenerative disorders like dementias, multiple sclerosis, Alzheimer's disease and Parkinson's disease (Maher, 2001; Benzi *et al.*, 1994). Neuronal loss, neuritic and cytoskeletal lesions represent the major dementia-associated abnormalities in Alzheimer's disease (de la Monte *et al.*, 2000). The loss of protein kinase C activity has been coupled to the severity of the damage although the functional relationship between oxidative stress, protein kinase C and cell death is unknown (Maher, 2001).

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Hypoxia leads to metabolic cellular processes in which oxidative species such as superoxide radical anions, hydrogen peroxide and lipid peroxides are generated intracellularly (Scandalios, 1997; Chen & Buck, 2000). These reactive species, if not eliminated, may damage DNA, proteins or membrane lipids and cause oxidative cell death. Endogenous antioxidative enzymes as well as endogenous small molecule antioxidants are required for cells to survive (Scandalios, 1997; Chen & Buck, 2000; Semenza 2005), while exogenous small molecule antioxidants have been shown to effectively prevent oxidative cell death in cultured cells (Busciglio & Yankner, 1995; Nakao *et al.*, 1996).

Hypoxic stress results in a rapid and sustained inhibition of protein synthesis that is partially mediated by eukaryotic initiation factor 2 alpha phosphorylation by the phospho-endoplasmic reticulum kinase (Blais *et al.*, 2004). Severe hypoxia has been shown to induce apoptotic cell death in developing brain neurons whereas mild hypoxia has been demonstrated to stimulate neurogenesis (Bossenmeyer-Pourie *et al.*, 2002). Hypoxia threatens brain function throughout the entire life span starting from early foetal age until death and although the physiological consequences of brain hypoxia are well documented, the molecular mechanisms involved are still not well understood (Zhu *et al.*, 2005; Rossler *et al.*, 2001; Semenza, 2006; Semenza, 2007).

It has been shown that hypoxia may have severe detrimental effects on most cells and especially on neuronal cells. Some studies have suggested that hypoxia can induce cellular adaptive responses that overcome apoptosis or cell death leading to reduced hypoxic cell injury, damage or cell death (Yun *et al.*, 1997; Banasiak *et al.*, 2000). These adaptive responses of cells to hypoxia may involve activation of some ion channels, as well as induction of specific gene expression which may help to suppress or limit the effects of hypoxia in these cells (Yun *et al.*, 1997). For example, adenosine triphosphate (ATP)-sensitive potassium ion (K<sup>+</sup>) channels are activated by hypoxia in some cortical cerebral neuronal cells, and this may play a role in cell survival during hypoxia (Yamada and Inagaki, 2002). This may explain why some cells may survive and adapt to the hypoxic environment than others. Also, hypoxia-induced basic fibroblast growth factor and nerve growth factor expression appear to be associated with prevention or delay of neuronal cell apoptosis in hypoxic conditions (Yun *et al.*, 1997; Banasiak *et al.*, 2000).

Hypoxia-induced oxidative stress can also cause neurite retraction leading to neurodegeneration, while hypoxia-like injury can cause neuronal loss. Both oxidative stress and hypoxic injury could contribute to neurodegeneration similar to that found in Alzheimer's disease (de la Monte *et al.*, 2000). It has been shown that ischemia results in severe focal and global damage of brain tissue accompanied by biochemical and molecular alterations, while hypoxia results in depletion of cellular and tissue energy and consequent death of the cells involved (Rodrigo *et al.*, 2005).

Neuronal pattern and pattern formation makes it possible for neurons to organize themselves into groups called nerve fibers that carry neural signals into specific areas of the body to and from the brain. These patterns and the pattern formation itself are very important in ensuring that specific signals are carried to specific areas for effective and efficient coordinated neural response (Golubitsky *et al.*, 2004). Neuronal fibers are arranged in bundles that receive and transmit related signals. These bundles form a neural tract e.g. the visual tract carries visual signals from the eyes to relay centers in the brain and back to the eyes for visual perception to occur (Cowan and Thomas, 2004). Asare *et al.* (1996) have shown that spatial differences exist in the distributional pattern of neurons in the superior frontal gyrus of 32 subjects who died of acquired immune deficiency syndrome. This gives support to the fact that, if there is a disruption in the tracts, the signal may not be properly coordinated and relayed for perfect response to occur. Genetic coding has been shown to play a role in the pattern formation in various part of the nervous system (Schmid *et al.*, 2000). The aim of the present work was to investigate the effects of hypoxia and opioid receptor agonists' treatment on the morphology of B50 neuronal cell lines cultured in hypoxia using neuronal pattern and pattern formation as a case study.

## **Materials and Methods**

### **Neuronal culture**

One group of B50 cells were cultured and maintained in a normal incubator (21% O<sub>2</sub>; 5% CO<sub>2</sub>) as control cells, with another batch of cells cultured in a hypoxic incubator (5% O<sub>2</sub>; 5% CO<sub>2</sub>) as hypoxic experimental cells. The cells were cultured in a 12-well culture plates for 48 hours and three highly potent opioid receptor agonists {DAMGO( $\mu$ ), DSLET( $\delta$ ) and ICI--199,441( $\kappa$ )}, were selected and administered to the cells as treatment against hypoxia for 48 hours at a concentration of 10 $\mu$ M, 50 $\mu$ M and 100 $\mu$ M.

## Morphological Studies

The frozen B50 cells were cells were raised in culture for 24, 48, 72, 96, 120 and 144 hours while 0 hour was regarded as the starting point of culture during splitting of cells for sub-culturing. At each stage of the experimental period, the cells were observed under the microscope, using same field morphological assessment in which the culture plates were examined from the centre to the sides in a quadri-point analysis method (Ellingson *et al.*, 2007; Sato and Momose-Sato, 2007), and any change in the morphology of the cells was noted. This was repeated three times for each experiment. Micrographs of the cells were taken at the different time intervals at a magnification of 200 times (x 200), to show the morphological changes that may have occurred between the normal and hypoxic cells in culture.

## Lactate dehydrogenase Assay

Lactate dehydrogenase (LDH) release which has been shown to be a reliable index of cellular injury (Zhang *et al.*, 2006), was used to assess the level of neuronal injury in normal, hypoxic and treated cells, using a LDH kit and procedure from Sigma. The working solution of LDH assay cofactor was prepared by adding 25ml of deionized, sterilized tissue culture water to bottle of lyophilized cofactors. The lactate dehydrogenase assay mixture was prepared by mixing equal amounts of LDH assay substrate, LDH assay cofactor and LDH assay dye solution. The LDH assay mixture was added at double the volume of the supernatant medium removed for assaying. The plates were covered with aluminum foil to protect it from light and incubated at room temperature for 30 minutes. The reaction was terminated by the addition of one tenth volume of 1N HCL solution to each well. The absorbance was measured at a wavelength of 490nm using Dynex MRX model of Micro plate reader and the result was calculated.

## Data Analysis

The parameters were assayed two times in triplicate in the normal, hypoxic and treated experimental groups of cultured B50 neuronal cells and the results are presented as mean  $\pm$  standard deviation (SD). A Students't-test was used to test the level of significance and a P-value less than 0.05 was considered to be significant. For multiple treatment data, One-Way Analysis of Variance (ANOVA) was used followed by Multiple Range Test post hoc subgroup testing to find the least significant difference (LSD) between the groups.

## Results

### Morphological Changes

The result showed morphological changes between the normal, hypoxic and treated B50 cells. The normoxic cells showed normal neuronal cell morphology (Figs.1 and 2), while the hypoxic non-treated cells showed groups of dead and degenerating cells (Figs.6 and 7). The hypoxic drug-treated B50 cells showed reduced cell death and healthier neuronal cells (Figs.8 and 9).

### LDH Release from Opioid agonists treated B50 neuronal cells cultured in hypoxia

The LDH leakage from normal B50 cell (100%) was significantly increased ( $p < 0.05$ ) when compared with untreated hypoxic cells (587%) and treated hypoxic B50 cells with different concentration of opioid agonists. The LDH released from B50 cells treated with 100 $\mu$ M MOR DAMGO (143%); 50 $\mu$ M DOR DSLET (140%); 100 $\mu$ M DOR DSLET (143%); 10 $\mu$ M KOR (113%) and 50 $\mu$ M KOR (109%) were significantly decreased ( $p < 0.05$ ) from untreated hypoxic cells in culture (Table 1). The LDH leakage from hypoxic untreated cells (587%) was 5 folds higher than that from normal cells, and cells treated with 100 $\mu$ M MOR DAMGO (143%); 50 $\mu$ M DOR DSLET (140%); 100 $\mu$ M DOR DSLET (143%); 10 $\mu$ M KOR (113%) and 50 $\mu$ M KOR (109%), and about three folds higher in cells treated with 10 $\mu$ M MOR (338%) and 100 $\mu$ M KOR (322%).

**LDH Release from Opioid agonists and antagonists treated B50 cells cultured in hypoxia.**

The LDH leakage from B50 cells in hypoxia treated with opioid agonists/antagonists showed that LDH released from the normal cultured B50 cells (100%) was significantly higher ( $p < 0.05$ ) in untreated hypoxic B50 cells (587%). The LDH release from hypoxic treated cells with different concentrations of opioid agonist in the presence of antagonist, showed that the LDH leakage from the hypoxic cells treated with opioid agonists/antagonists was increased between 2-8 folds when compared with the normal cultured B50 cells while the LDH release was about the same as in those treated with 50 $\mu$ M DOR/ICI (416%), 50 $\mu$ M KOR/NOR (529%) and 100 $\mu$ M KOR/NOR (665%) when compared with untreated hypoxic cells (Table 2).

**Pattern formation**

The results from the morphological study showed the B50 neuronal cells in the normal incubator with normal neuronal pattern and tract formation resembling those nerve fibres in normal neuronal development in the body (Figs.3, 4 and 5), when compared to the disoriented pattern found in B50 cells cultured under hypoxia (Figs.6 and 7). The B50 neuronal pattern and pattern formation was shown to be greatly improved in cells treated with opioid receptor agonists (Figs. 8 and 9), when compared to the abnormal pattern found in hypoxic cells (Figs. 3, 4 and 5).

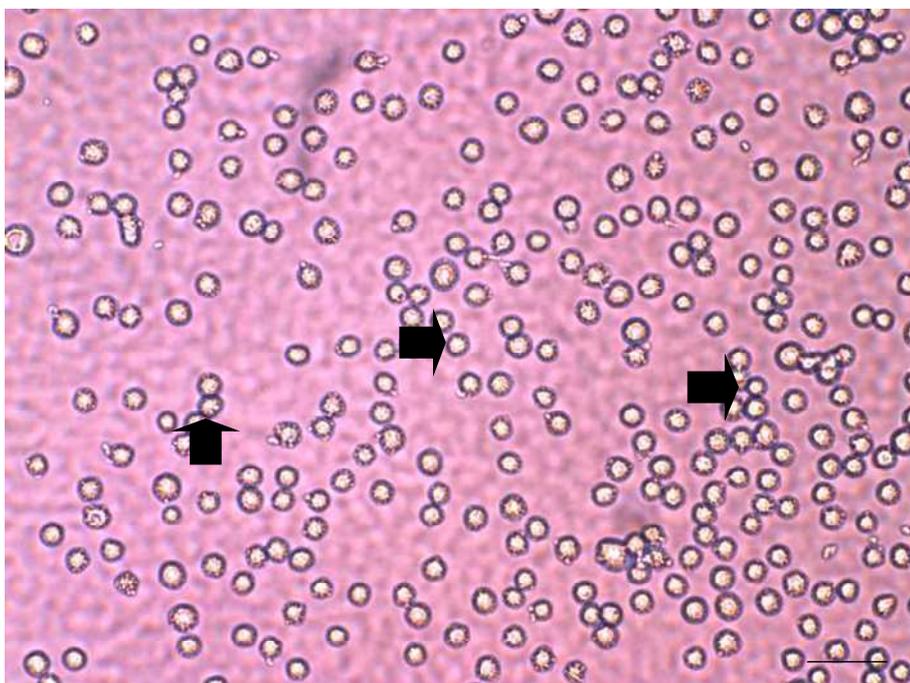


Plate 1: Representative of B50 cells at 0hrs with normal B50 cells (arrow) at the point of starting the culture at 21% O<sub>2</sub> and 5% CO<sub>2</sub>. B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

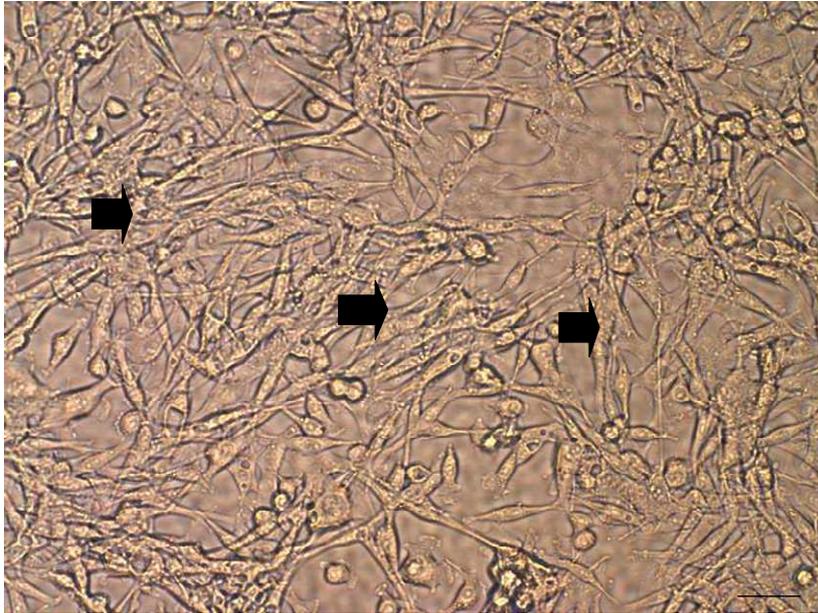


Plate 2: Representative of B50 cells at 48hrs of normal culture (21%O<sub>2</sub> and 5% CO<sub>2</sub>) with B50 cells (arrow). B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with IBM Image Solutions®. Scale bar =5mmx40 magnification.

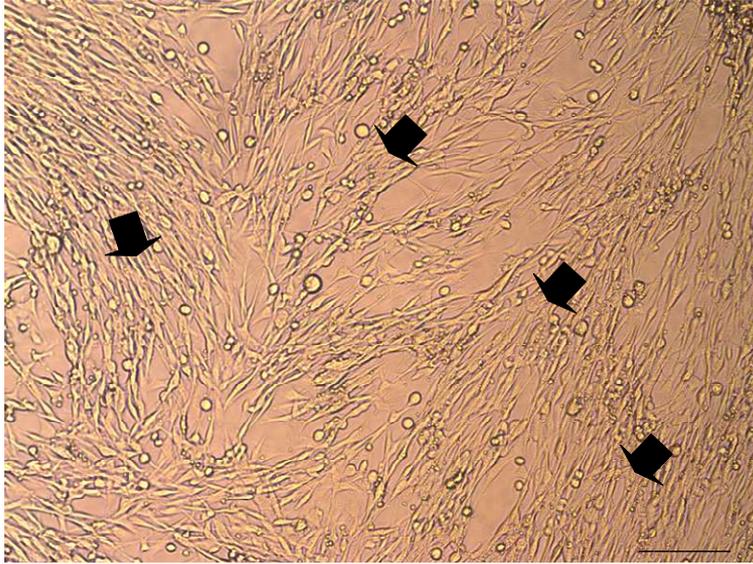


Plate 3: Representative of normal B50 cells at 48hrs of culture (21%O<sub>2</sub> and 5% CO<sub>2</sub>) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx20 magnification.

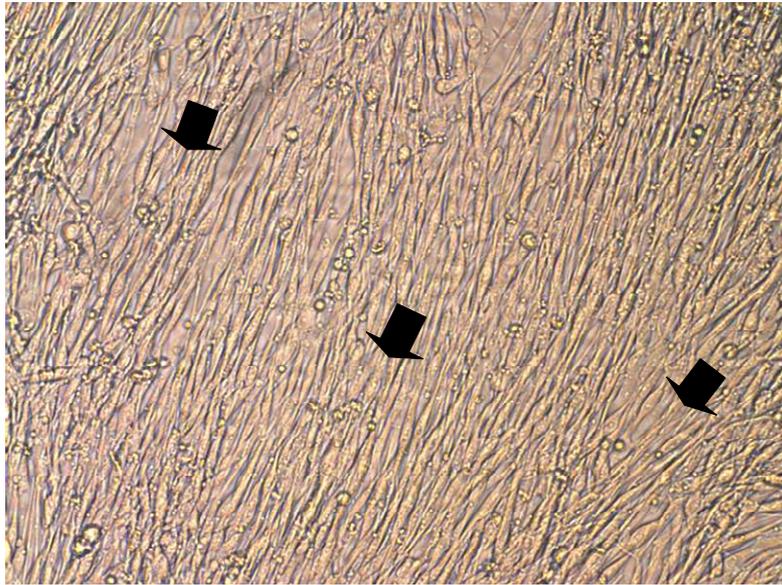


Plate 4: Representative of normal B50 cells at 96hrs of culture (21%O<sub>2</sub> and 5% CO<sub>2</sub>) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

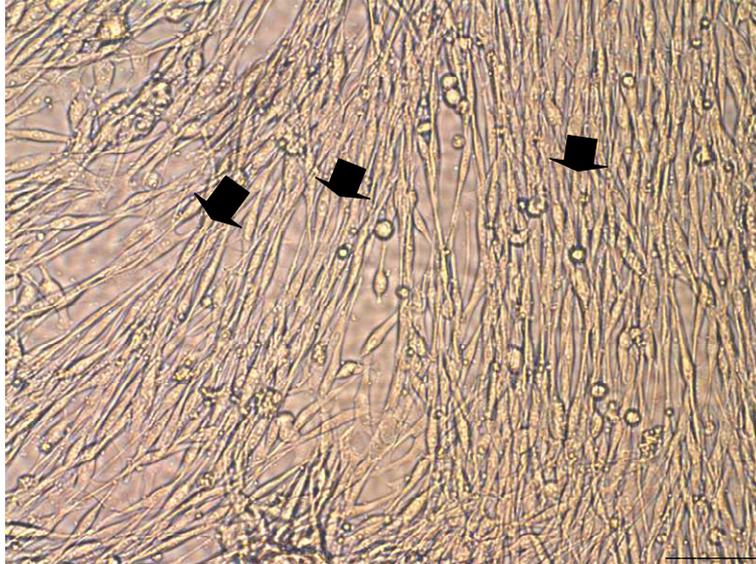


Plate 5: Representative of normal B50 cells at 96hrs of culture (21%O<sub>2</sub> and 5% CO<sub>2</sub>) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification

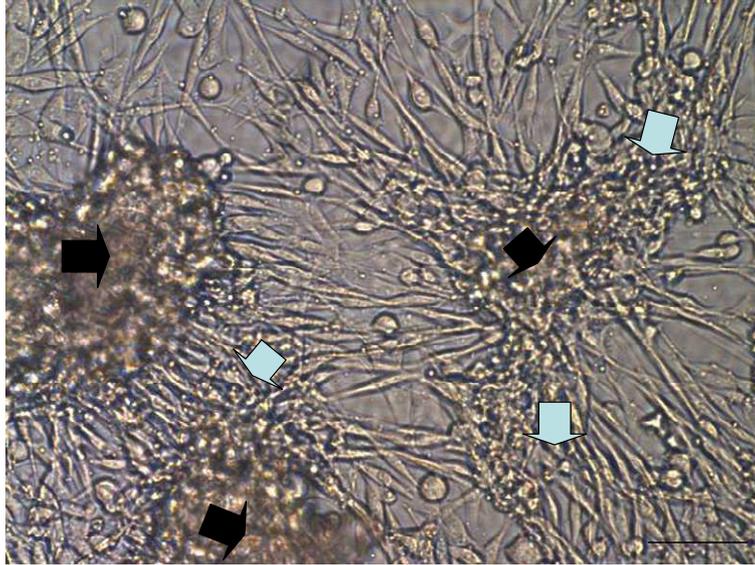


Plate 6: Representative of hypoxic B50 cells at 96hrs of culture (5%O<sub>2</sub> and 5% CO<sub>2</sub>), with groups of degenerating cells (black arrow) and altered pattern of cell arrangement (blue arrow). Groups of degenerating and altered nerve arrangement were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

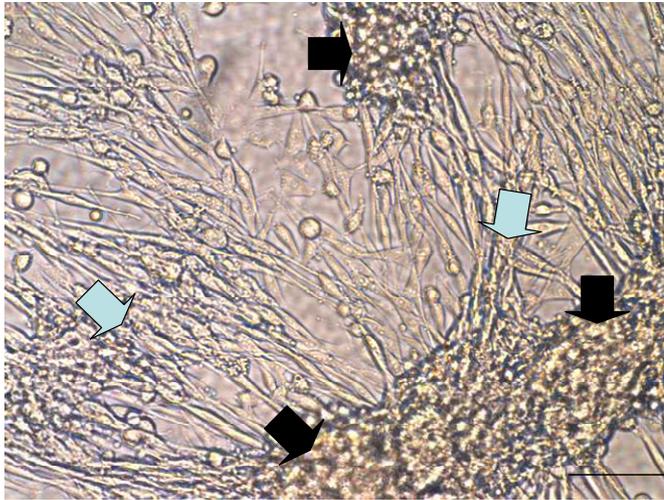


Plate 7: Representative of hypoxic B50 cells at 96hrs of culture (5%O<sub>2</sub> and 5% CO<sub>2</sub>), with groups of degenerating cells (black arrow) and altered pattern of cell arrangement (blue arrow). Groups of degenerating and altered nerve arrangement were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

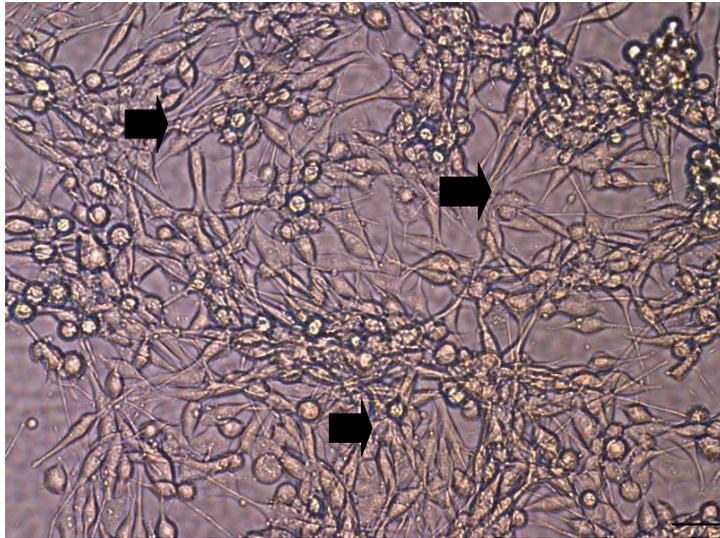


Plate8: Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 100  $\mu$ M DAMGO against hypoxia for 48hrs making-up 96hrs of culture (5%O<sub>2</sub> 5% CO<sub>2</sub>), showing many normal cells (Black arrow). The cell was observed in six different cultures with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

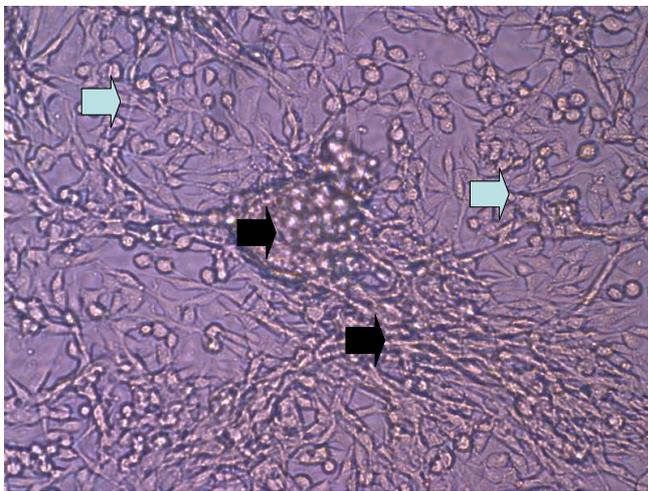


Plate9: Representative of B50 cells pre-treated with 100  $\mu$ M DSLET against hypoxia for 96hrs of culture (5%O<sub>2</sub> 5% CO<sub>2</sub>), showing few degenerated cells (Black arrow) and some normal cells (Blue arrow). The cells was observed in six different cultures with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

**Table1: The effect of opioid agonist treatment on LDH release in cultured B50 cells in hypoxia**

| Type of Treatment  | Measured optical density(OD) | Percent of control (% of Control) |
|--------------------|------------------------------|-----------------------------------|
| Normal Culture     | 0.207±0.101                  | 100±10.1                          |
| Hypoxia no Drug    | 1.214±0.511                  | 587±51.1*                         |
| DAMGO(μ)10μM       | 0.699±0.421                  | 338±42.1* <sup>#</sup>            |
| DAMGO(μ)50μM       | 0.344±0.210                  | 167±21.0 <sup>#</sup>             |
| DAMGO(μ)100μM      | 0.296±0.112                  | 143±11.2 <sup>#</sup>             |
| DSLET(δ) 10μM      | 0.346±0.201                  | 167±20.1 <sup>#</sup>             |
| DSLET(δ) 50μM      | 0.290±0.112                  | 140±11.2 <sup>#</sup>             |
| DSLET(δ) 100μM     | 0.296±0.221                  | 143±22.1 <sup>#</sup>             |
| ICI-199441(κ) 10μM | 0.234±0.221                  | 113±22.1 <sup>#</sup>             |
| ICI-199441(κ) 50μM | 0.224±0.223                  | 109±22.3 <sup>#</sup>             |
| ICI-99441(κ)100μM  | 0.666±0.251                  | 322±25.1* <sup>#</sup>            |

The effect of opioid agonist treatment on LDH release from B50 cells cultured in hypoxia (5% O<sub>2</sub>; 5% CO<sub>2</sub>), using LDH assay. Cells were cultured for 48hrs and then treated with different concentrations of opioid agonists for 48hrs for a total of 96hrs of culture. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells (21% O<sub>2</sub>;5% CO<sub>2</sub>) was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; \*P<0.05 versus untreated hypoxic cells; <sup>#</sup>P<0.05 versus agonist treated hypoxic cells; Student's t-test).

**Table 2: The effect of opioid agonist/antagonist treatment on LDH release from cultured B50 cell in hypoxia**

| Type of Treatment       | Measured optical density(OD) | Percent of control (% of Control) |
|-------------------------|------------------------------|-----------------------------------|
| Normal Culture          | 0.207±0.101                  | 100±10.1                          |
| Hypoxia no Drug         | 1.214±0.511                  | 587±51.1*                         |
| DAMGO(μ)10μM            | 0.699±0.421                  | 338±42.1*                         |
| DAMGO/CTAP10μM          | 0.726±0.335                  | 351±33.5*                         |
| DAMGO(μ)50μM            | 0.344±0.210                  | 167±21.0                          |
| DAMGO/CTAP50μM          | 0.535±0.252                  | 259±25.2* <sup>#</sup>            |
| DAMGO(μ)100μM           | 0.296±0.112                  | 143±11.2                          |
| DAMGO/CTAP100μM         | 0.752±0.305                  | 364±30.5* <sup>#</sup>            |
| DSLET(δ) 10μM           | 0.346±0.201                  | 167±20.1                          |
| DSLET/ICI-174864 10 μM  | 0.530±0.256                  | 256±25.6* <sup>#</sup>            |
| DSLET(δ) 50μM           | 0.290±0.112                  | 140±11.2                          |
| DSLET/ICI-174864 50 μM  | 0.859±0.345                  | 416±34.5* <sup>#</sup>            |
| DSLET(δ) 100μM          | 0.296±0.221                  | 143±22.1                          |
| DSLET/ICI-174864 100 μM | 0.644±0.225                  | 312±22.5* <sup>#</sup>            |
| ICI-199441(κ) 10μM      | 0.234±0.221                  | 113±22.1                          |
| ICI-199441/NOR 10μM     | 1.698±0.501                  | 821±50.1* <sup>#</sup>            |
| ICI-199441(κ) 50μM      | 0.224±0.223                  | 109±22.3                          |
| ICI-199441/NOR 50 μM    | 1.093±0.552                  | 529±55.2* <sup>#</sup>            |
| ICI-99441(κ)100μM       | 0.666±0.251                  | 322±25.1                          |
| ICI-199441/NOR 100 μM   | 1.375±0.446                  | 665±44.6* <sup>#</sup>            |

The effect of opioid agonist/antagonist treatment on LDH release from B50 cells cultured in hypoxia (5% O<sub>2</sub>; 5% CO<sub>2</sub>), using LDH assay. Cells were cultured for 48hrs and then treated with different concentrations of opioid agonists for 48hrs for a total of 96hrs of culture. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells (21% O<sub>2</sub>;5% CO<sub>2</sub>) was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; \*P<0.05 versus untreated hypoxic cells; <sup>#</sup>P<0.05 versus agonist/antagonist treated hypoxic cells; Student's t-test).

## Discussion

The results presented in this study showed hypoxia-induced changes in the neuronal B50 cell morphology, pattern and LDH of neuronal viability. The results showed that hypoxia has an effect on neuronal pattern and pattern forming abilities of B50 neuronal cells when compared to normal B50 cells in culture. The neuronal pattern is very important for an organized and integrated neuronal activity which could be disrupted if the pattern is disorganized or disoriented as shown in hypoxic cells in this study. This is because the normal neuronal organization involves the grouping of nerve fibers to form nerve bundles of which functionally related nerve bundles ultimately become grouped together and form specific nerve tracts. These linkages of neurons are possible through synapses where signals are passed between cells. The disruption of the neuronal patterns as shown during hypoxia in this study, indicate that hypoxia could result in the alteration of signal transmission between neuronal cells since some of the neuronal cells are dead and degenerating. These changes in the pattern could also explain the changes in the morphology since the neuronal pattern of arrangement is a part of neuronal morphology.

The changes in morphology, neuronal pattern and LDH that occur during hypoxia in B50 cells in culture were in agreement with the study by Titus *et al.* (2007), which showed that simulated hypobaric hypoxia, resembling that found in high altitude hypoxia, severely affects the morphology of the central nervous system (CNS) and results in several physiological changes. They showed that these effects specifically within the hippocampus may closely be associated with learning and memory and proposed that insult to this region also affects cognition.

The B50 neuronal cell degeneration in hypoxic cultures may be linked to the loss of neuronal cells and tissues which invariably would result in defects in neuronal activities. These B50 neuronal cell losses and loss of normal neuronal pattern results in loss of neuronal mass, which could lead to loss of nervous system coordination of activities and actions from the cellular level to the whole organism. The neuronal loss may affect cognition, behavioral activities, learning, memory and may even lead to physical defect depending on the area of the brain affected by the degeneration. Studies by Shukitt-Hale *et al.* (1994), had suggested that rapid or prolonged exposure to hypobaric hypoxia (HBH), is associated with psychomotor and cognitive impairments.

The results presented in this study which showed changes in the characteristics of neuronal B50 cells in hypoxia compared to the normal B50 cells support the work of Titus *et al.* (2007), and Shukitt-Hale *et al.* (1996). They showed that there were structural changes in the neuronal cells of both human and animal models exposed to hypoxia. These structural and morphological changes can lead to defects in memory, cognitive behaviour and loss of coordinated responses to stimuli.

The result with the B50 neuronal cells showed that cell damage increases with the increase in the exposure time to hypoxia. Thus, the more time the cells are exposed to the hypoxic environment the greater the damage to the B50 cells. This is in agreement with the findings of Titus *et al.* (2007), which showed that the damage to cells increases with the level of exposure to an oxygen-deprived environment and that the number of damaged cells increases following increase in altitude. Clausen *et al.* (2005) correlated the hippocampal morphological changes and memory dysfunction, cortical lesion volume and regional hippocampal morphological changes after controlled cortical contusion (CCC) injury in rats, and showed morphological changes in CA1, CA3, and hilus of the dentate gyrus in severely injured rats.

Sousa *et al.* (2000) have demonstrated that stress-induced cognitive deficits in rats do not correlate with hippocampal neuronal loss when they evaluated the effects of chronic stress on hippocampal dendrite morphology, volume of mossy fibre system, and number and morphology of synapses between the mossy fibres. They found profound changes in the morphology of the mossy fibre terminals and significant loss of synapses were detected in stress-induced cognitive conditions (Sousa *et al.*, 2000). The study by Sousa *et al.* (2000) showed that stress-induced morphological changes in the hippocampus could not be correlated with cognitive changes in rat.

Yoshimura *et al.* (2006) have shown that neuronal polarity is essential for both structurally and functionally unidirectional signal flow from dendrites to axons. The initial event in establishing a polarized neuron is the specification of a single axon and early in neuronal development, one small neurite becomes differentiated from other neurites to form an axon. The ability of neuronal cells to proliferate, differentiate and polarize is essential for organization of the nervous system and cultured hippocampal neurons which develop a single long axon and several shorter dendrites that maintain their structural characteristics at the molecular level have been used as a model for neuronal polarization (Yoshimura *et al.*, 2006). During maturation, hippocampal neurons dramatically change their morphology and differentiate into a single axon and many dendrites which form synaptic contacts and establish a neuronal network (Yoshimura *et al.* 2006; Dotti *et al.*, 1988). It is these neuronal networks that finally joined together into a specific pattern to form neuronal bundles that ultimately form neural tracts.

The result as presented in this study showed that hypoxia affected neuronal pattern and pattern formation in B50 cells in hypoxia when compared with the normal B50 cells in culture. This disorganization of the neuronal pattern could be as a result of the reduction and disorientation of the proteins necessary for cellular signaling through neuronal cell proliferation and differentiation. Of relevance are the findings of Zhong *et al.* (2007), who showed that Raf serine/threonine kinases play important role in nervous system development and when these molecules were conditionally eliminated, the result showed markedly reduced phosphorylation of ERK in neural tissues which led to growth retardation. This supports the finding in this present study.

In conclusion, the data presented here showed that hypoxia had an effect on B50 neuronal cell morphology, pattern and pattern formation which could have a deleterious effects on neuronal structure and morphology which would result in overall functional impairment of the nervous system in terms physical activities, cognitive abilities, learning and memory functions even though this may depend on the parts of the brain that are affected. This could be correlated with changes in the morphology of the brain as seen in the study with other neurodegenerative conditions like Stroke, Alzheimer's disease (AD) and Parkinson's disease (PD). The changes in morphology, neuronal pattern and LDH release indicate that hypoxia induced morphological and cellular changes in B50 cells in hypoxia and B50 neuronal cells are susceptible to damage and injurious effects of hypoxia as most brain cells while opioid agonists have some potential benefits in the treatment of hypoxia-induced changes in neuronal B50 cells in culture.

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