Available online at www.ijmrhs.com

ISSN No: 2319-5886

International Journal of Medical Research & Health Sciences, 2020, 9(6): 13-21

The Impact of Methotrexate on Neural Stem Cells and the Protection Provided by Cells in the Neurogenic Niche

Anmar Alharganee[1*], Haydar Alabedi[1], Marwah Abdulrahman[2] and Peter Wigmore[3]

[1] Department of Oncology, Nottingham University Hospital/QMC, Baghdad Medical City Complex, Iraq

[2] Specialized Center of Endocrinology and Diabetes, Ministry of Health Iraq, Iraq

[3] Medical School, Nottingham University Hospital/QMC, Iraq

**Corresponding e-mail: dr_harganee@yahoo.com*

ABSTRACT

While the survival rates for cancer have dramatically increased with the use of chemotherapy, substantial numbers of patients complain of cognitive impairments associated with this treatment. These symptoms are known as "chemobrain" and include loss of concentration and memory associated with poor work performance. One cause of chemobrain appears to be a decrease in the neural stem cell proliferation essential for adult hippocampal neurogenesis. This produces new neurons required for memory consolidation. Neural stem cell proliferation occurs in a stem cell niche within the sub granular zone (SGZ) of the dentate gyrus. Recent in vivo studies have shown that in the SGZ, neural stem cells associated with blood vessels survive chemotherapy better than those not in contact with the vasculature. Our in vitro study tested the effect of Methotrexate (MTX) on dividing neural stem cells and brain endothelial cells separately and when co-cultured in contact with one another. The results, using MTT assays, show that neural cells are significantly more sensitive to MTX than endothelial cells. Marked neural cells were then cultured on a mono layer of either endothelial or non-neural cells (3T3 fibroblasts) and treated with MTX. Co-culture with endothelial cells offered significant protection from MTX compared with culture with non-neural cells or neural cells on their own. This in vitro model replicates in vivo observations and can be used to study the protective role of endothelial cells on neural stem cells. An understanding of this may be used to preserve hippocampal neurogenesis and reduce the incidence of chemobrain.

Keywords: Methotrexate, Stem cells, Chemobrain, Endothelial cells

INTRODUCTION

Methotrexate (MTX)

This drug is an antimetabolite and anti-folate drug with a molecular weight of 454.44 g/mol that acts as an anti- proliferative agent by antagonizing the effect of folic acid and the ability to bind to dihydrofolate reductase. Furthermore, MTX may inhibit thymidylate synthease resulting in a transmythelation of proteins, other mechanism involve its effect on de novo purine bio-synthesis by inhibiting the enzyme aminoimidazole carboxamidoneucliotide, essential enzyme involved in purine bio synthesis pathway. MTX is used in the adjuvant and palliative treatment of many malignancies including breast, acute leukemia, lymphomas, osteosarcomas, head and neck tumors and other cancers. In addition, (MTX) is used in the treatment of other non-malignant diseases such as rheumatoid arthritis and dermatological illnesses like psoriasis [1,2]. MTX is one of the common drugs used in the treatment of breast cancer however studies have found a number of adverse effects which include memory impairments in patients.

Animal models which have looked at the impact of MTX on cognition have found poor performance in object recognition tasks and conditional emotional response tasks to location and these results indicate that MTX is impairing spatial memory, a hippocampal dependent function [3]. A number of clinical studies have concluded that patients treated with weekly low-dose MTX may experience symptoms of headache, dizziness, memory loss and cognitive

impairments [4-6]. Patients receiving a high-dose of intravenous MTX may suffer from stroke like syndrome and focal neurological symptoms including aphasia, hemiparesis, encephalopathy and seizures [7,8]. Besides, these symptoms generally progress days to weeks after starting MTX therapy. Brain imaging using MRI can detect these neurological changes by showing hyper intense foci on FLAIR and diffusion-weighted sequences [9].

Delayed complications are common with MTX treatment as studies have suggested that patients suffer from symptoms of leukoencephalopathy months to years after receiving intravenous MTX and these symptoms may present clinically as a slow onset cognitive dysfunction or personality changes [10]. Cranial irradiation therapy combined or concurrent with MTX has been proved, by brain imaging of these patients, to exacerbate these cognitive deficits and may progress to severe weaknesses, dementia, loss of consciousness and death. Unfortunately, no effective treatment had been identified to manage these symptoms, yet *in vivo* studies had been suggested as a fast and more detailed way to investigate these symptoms [11].

Animals treated with a combination of MTX and 5 FU suffered from a significant decline in memory and performance in cognitive tasks [12]. Investigations have shown that rats when treated with MTX as a monotherapy, experience a decline in behavioral tasks that can be interpreted as deficits in hippocampal and frontal cortex functions. On the other hand, cognitive deterioration can be correlated with changes in long term potentiation and conduction accompanied with a reduction in hemogenesis [13-15]. In addition, animals treated with MTX were noticed to have structural and functional neurological changes demonstrated by imaging techniques. These changes are thought to be equivalent to 4 years increase in ageing [16].

Both *in vitro* and *in vivo* studies indicate that many chemotherapeutics including MTX could exert an effect on both normal and cancer cells. Dividing neural stem and precursor cells are found in the hippocampus and walls of the lateral ventricle. These are involved in the process of adult neurogenesis and generate new nerve cells in the denate gyrus and the olfactory system respectively. These cells appear to be a particularly susceptible cell types to chemotherapeutics [17,18]. MTX may also affect oligodendrocyte precursor cells, which are essential for preserving white matter tracts, also non dividing mature oligodendrocytes leading to demyelination and changes in conductance [19]. In contrast, mature astrocytes have been shown to be less affected by chemotherapy drugs [15]. The differences in cell sensitivity may be associated with cellular variations in mitochondrial respiration or different metabolic pathways within each cell.

Aims and Objectives

To quantify whether different components of the neurogenic niche-neural stem cells, astrocytes, 3T3 fibroblasts and endothelial cells show differential sensitivity to MTX

To determine if other cellular components of the neurogenic niche (astrocytes or endothelial cells) are able to protect neural cells from MTX

An understanding of how neural cells can be protected may enable the development of treatments which would benefit patients where a decrease in hippocampal neurogenesis caused by MTX is causing cognitive problems

MATERIALS AND METHODS

Growth Media Preparation

RPMI-1640 growth media without supplements, foetal bovine serum (FBS), 200 mM L-glutamine, penicillin G/Streptomycin antibiotics mixture (100 units/ml and gentamycin 25 mg/ml respectively, sodium pyruvate and non-essential amino acids and vitamins which were obtained in a sterile form. Then 150 ml of RPMI were removed and 10 ml of FBS were added, then L-glutamine, sodium pyruvate (Sigma life science S8636, Lot RNBD3337), vitamins, non- essential amino acids (Gibco of life technology Ref: 11140-035, Lot 1605380) were added to RPMI and mixed well by inverting the bottle several times , a small aliquots was taken in a sterilin tube and placed in the incubator as a sterility check. Then 10 ml penicillin G/Streptomycin antibiotics mixture and 250 μl of gentamycin were added.

Bringing Cell Lines Up from Liquid Nitrogen

Vials of frozen cells stored in nitrogen were defrosted by heating rapidly in a 37°C water bath with gentle shaking the vial (<1 min). 20 ml of growth medium was added to a 30 ml Universal tube. Before opening the tube, the outside of

the vial was cleaned with 70% IMS. When the ice crystals melted, the thawed cells were gently pipetted into a 30 ml centrifuge tube, before centrifuging at approximately 1500 rpm for 5 min. the supernatant was removed without disturbing the cell pellet. 5 ml of medium was added and mixed with the pellet and transferred into the appropriate culture flask. The growth medium was changed after approximately 24 hours, and the cells were passaged when confluent (usually after 3 days). Cells removed from frozen storage were grown-up for two passages before use in experimental studies.

Experimental Preparation of Cell Lines

Cells were cultured in 75 cm^2 polystyrene flasks fitted with 0.22 µm filter caps (Nunc) that contained 20 ml of RPMI growth medium, work field and gloves were sterilized with 70% IMS. Growth medium were removed by aspiration and cell monolayers were disrupted by the addition of 3ml of 10% trypsin/EDTA in PBS (1 ml EDTA and 9 ml PBS), after that the flask was incubated at 37°C for 5 minutes. 7 ml of fresh medium were added to the flask, the resulting suspension was transferred to a sterile 30 ml Universal tube and centrifuged at 150 g for 5 minutes then the supernatant layer was removed and 3 ml fresh media was added to the pellet, the resulting suspension was transferred to a fresh cell culture flask with 20 ml fresh growth medium according to the ratio of splitting for different cells.

Immunocytochemistry

Primary astrocytes were plated onto poly L-lysine (100 mg/ml, Sigma Aldrich, USA) coated cover slips and grown until confluent. After aspiration of the growth medium the cells were washed 3 times with PBS and fixed in 4% paraformaldehyde for 5 minutes and again washed with PBS 3 times (5 minutes each). Cultures were blocked with 5% BSA (Bovine Serum Albumine)+20 µl/ml of goat serum, 0.3 Triton x-100 in PBS for 1 hour then incubated with primary antibody, polyclonal anti-GFAP, (DAKO) diluted 1:500 with blocking buffer and 100µl of this dilution was added to each well and incubated overnight 4ºC. Goat anti-rabbit (Biotin) diluted 1:1 with diluents and 100 µl was added to each well and incubated at 37ºC for 1 hour. After washing with PBS for 3 times the slides were stained with DAPI (1:1000) for 5 minutes, washed again with PBS/3 times and mounted with DABCO. The slides were seen by Nikon microscope and SPOT insight camera. Controls in which the primary antibody was omitted had no apparent staining.

MTT Assay

Cells were cultured in 24-well plates (Coaster, USA) overnight at a density of 1 × 105 cell/well) and then exposed to a different concentration of MTX (2 µM, 5 µM, 7 µM and 10 µM) for 48h. The medium was removed and cells were treated with 50 µl of MTT (Sigma Aldrich, USA-5 mg/ml in PBS) followed by incubation in 5% CO_2 at 37°C for 30 minutes. 1000ul of DMSO (Sigma Aldrich, USA) were added to stop the reaction, 200 µl aliquots were taken from each well and transferred into a 96 well plate. The readings were repeated 3 times and the final product of MTT solubilized formazen was measured by spectrophotometer under the absorbance of 570 nm to conclude the density of viable cells in each well that is based on the ability of viable cells to reduce a yellow tetrazolium salt metabolically to purple formazan [20].

Co-culture of N2A Cells on Monolayers of Different Cell Types

3 cell lines were used in this experiment including the mouse neuroblastoma cell line N2A from the Public Health England Culture Collection [21], human brain micro vascular endothelial cell lines from ScienCell Research Laboratories [22] and mouse fibroblast cell lines 3T3 [23]. Endothelial or 3T3 cell lines were grown in T75 flask then tripsynised and plated into a 4-well chamber slide (Lab Tek-Nuna) and grown overnight. N2A cells were stained before co-culture using PKH26 red fluorescent cell linker (Sigma Aldrich, USA). Stained single cell suspensions of N2A were plated on top of confluent endothelial or 3T3 cells at a low density (1 × 104 cell/ml) for 24 hours. Following this 1ml of 2 µM MTX was added to the co culture and incubated for 48 hours. Growth medium was removed and cells were fixed in 4% paraformaldehyde. Cells were washed with PBS and cell nuclei visualized after staining with DAPI. Cells were washed with PBS (every 5 minutes)/3 times. The plastic rim was removed and the slides were coversliped and mounted with DABCO. All slides were viewed using a Nikon EFD-3 fluorescence microscope and photographed with a Spot Insight QE camera with Spot advanced software (Image Solution Ltd). Fields were selected randomly and cell counting was done blindly. In each experiment 4 wells were used as N2A, N2A+MTX, N2A co cultured and N2A co cultured +MTX.

Statistical Analysis

Data were collected from three or more independent experiments (using different passages of cells) and are expressed in form of the mean ± S.E.M. Statistical analysis was carried out using Prism (6.0, Graph Pad software, San Diego California, USA). One way ANOVA and t-tests were used to compare groups. Parametric statistical analysis was used if the data was checked to be normally distributed (D' Agostino Pearson omnibus normality test) and Statistical significance was proven at $p<0.05$.

RESULTS

Immunostaining Analysis of C6 Cells

The astrocyte marker GFAP was immunostained to confirm the phenotype of primary hippocampal cells. Morphologically Long (branched) and short (un-branched) cells were noticed after 2 days cells culture. About 95% of the cells were stained positive for GFAP and the immunocytochemistry for GFAP showed that the staining evenly distributed through the cytoplasm of cells. This result confirmed that the primary cells isolated from the hippocampus were astrocytes (Figure 1).

The Sensitivity of Different Cell Types within the Hippocampal Stem Cell Niche to Methotrexate

Different concentrations of MTX were tested on various cell lines within the hippocampal stem cell niche and a non-neural cell line (3T3) for comparison. The cell lines used were C6, N2A, brain endothelial and 3T3 fibroblast. The concentrations of MTX used were (2, 5, 7 and 10) µm. the effect of MTX on cell lines were measured by MTT assay.

From the results a significant effect of 2 µm MTX was observed on all cell lines. However, the sensitivity differed between cell types. C6, N2A and 3T3 fibroblasts all showed a substantial sensitivity to MTX with 2 µm MTX reducing the viability by over 50%. In contrast the endothelial cells that showed significantly more resistance and maintained viability to at least 80% of untreated cells (Figure 2).

Co-culture of Different Components of the Stem Cell Niche with Neural Cells to Determine if Any Components can Protect Neural Stem Cells from MTX

From previous results a concentration of 2 µm has a major effect on N2A cells but significantly less effect on endothelial cells. From these results using 2 µm of MTX on co-cultures of N2A cells with endothelial cells should make it possible to test the role of endothelial cells in protecting N2A from the effect of MTX. Endothelial cells were cultured as monolayer and marked N2A cells were seeded on to them prior to MTX therapy (Figure 3).

N2A cells showed no significant increase in cell number when cultured on endothelial cells (N2A and N2A+ENDO groups). As previously shown by MTT assay, the number of N2A cells was considerably decreased when treated with 2 µm MTX (N2A+MTX group). By contrast, N2A cells co cultured with endothelial cells showed significantly better survival when treated with MTX (N2A+ENDO+MTX and N2A+MTX groups). This suggests that the presence of endothelial cells has a protective role on N2A cells treated with MTX.

To test if this effect was specific to endothelial cells or a general effect of co-culturing N2A cells with another cell type, N2A cells were co-cultured with the non-neural 3T3 cell line. In contrast to the results with endothelial cells, co culture of N2A cells with 3T3 fibroblasts had no significant effect on the viability of N2A cells when treated with 2 µm MTX (Figure 4).

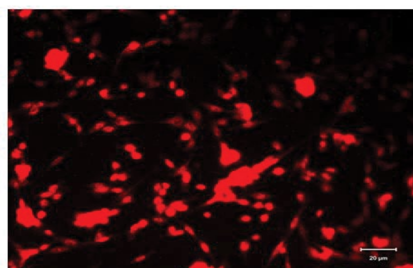

Figure 1 Immunoconformation of C6 glioma phenotype; Immunostaining micrograph showing the expression of GFAP in primary hippocampus cells monolayer grown on coverslips; Scale bar=20 µm

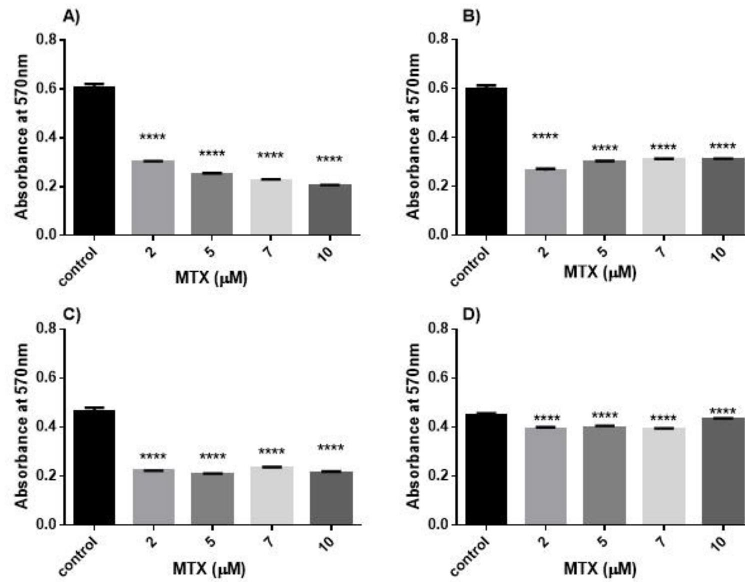

Figure 2 Effect of MTX on C6 glioma (A) N2A neuroblastoma (B) 3T3 fibroblast (C) and endothelial (D) cell lines; Assessed by optical density at 570 nm wavelength (normalized as a proportion of the control (0 µm of MTX) using an MTT assay; Confluent cells were exposed to a different concentrations of MTX (2, 5, 7 and 10) µm for 48 hours; Data shown as mean value ± SEM (N= 3 experiments); All were analyzed by One way ANOVA, where p<0.05

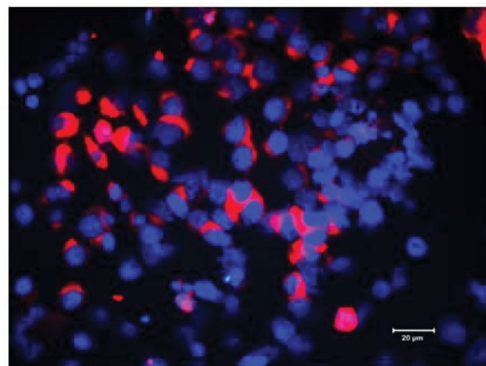

Figure 3 Co-culture N2A cells (red) on a monolayer of endothelial cells. Cell nuclei stained with DAPI (blue), N2A cells marked with PKH26; Scale bar=20 µm

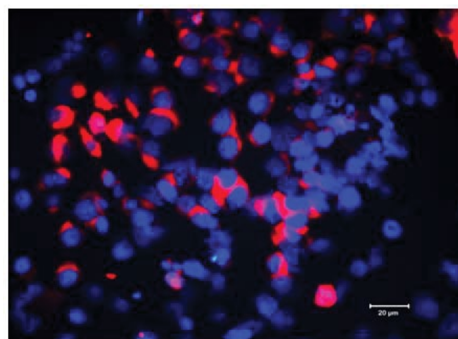

Figure 4 Effect of Endothelial (A) and 3T3 Fibroblasts (B) on the Survival of N2A Cells cultured on single and co-culture in presence or absence of (2 µM MTX); Significant protection can be detected in N2a cells from co-culture with endothelial cells after 48 h of exposure to MTX. In contrast, no protection can be observed in those co-cultured with 3T3 cells; Data shown as mean value ± SEM (N=3 experiments); All were analyzed by one way ANOVA, where p<0.05

DISCUSSION

This project follows on from the demonstration *in vivo* that MTX reduces cells proliferation in the SGZ of the dentate gyrus and this is associated with cognitive impairments [12]. This work on rats showed that proliferating cells in contact with blood vessels in the SGZ appeared to be resistant to chemotherapy and continued to divide after treatment (Maqbool personal communication). As the decline in hippocampal neurogenesis is one of the most likely causes of cognitive impairments in patients treated with chemotherapy, an understanding of how proliferating neural stem cells could be protected might provide a treatment to reduce this effect. In this study we wished to develop an *in vitro* model of the interactions between dividing neural cells and other cellular components of the neurogenic niche in terms of their response to a commonly used chemotherapy agent, MTX which has been associated with cognitive impairments in patients. To do this we tested the *in vitro* sensitivity of different cell types found in the neurogenic niche to MTX and then assessed if co-culture between N2A and other cell types provided protection from MTX.

The Sensitivity of Different Cell Types within the Hippocampal Stem Cell Niche to Methotrexate

The effects of MTX on cell lines within the neurogenic niche were found to differ between cell types. N2A cells showed a great sensitivity to MTX with the lowest concentration used reducing cell viability to less than 50% (Figure 2B). This result is consistent with results from *in vivo* studies where animals treated with MTX showed a reduction in the proliferation of neural stem cells in the SGZ of the hippocampus [3,13]. Animal studies by our research team have also found that treatment with chemotherapy does not reduce blood vessel density in the dentate gyrus (Maqbool personal communication). The non- neural 3T3 fibroblast cells were sensitive to chemotherapy as found in previous studies [4]. The C6 astrocyte cell line was also found to be sensitive to MTX. This was surprising as C6 cells have been found to be resistant to the chemotherapy agent 5Fluorouracil (5FU) (Entedhar personal communication) and as such possibly providing a protective role to proliferating neural cells. A range of high and low doses of MTX were used to determine the effect on C6 cells and at all doses, these cells were found to be highly sensitive to MTX, in contrast, to the results from 5-FU. This might be an interesting area to explore in future work as these two drugs are frequently used in combination regimes in breast cancer patients and their use together may exacerbate the cognitive side effects of treatment [24].

In contrast to the results from C6 cells endothelial cells were more resistant to MTX. This is similar to previous results by our research group demonstrating their resistance to 5FU (Entedhar personal communication). The relative resistance of endothelial cells makes it possible that they may be able to protect adjacent neural cells from chemotherapy. This was tested using the proliferating neural cell line N2A cells which we have demonstrated to be sensitive to MTX. This resistance to MTX comes along side with a study concluded that endothelial cells within coronary arteries were resistant to high and low dose MTX [25]. All cells viability was measured by MTT assay, and this fluctuation in cellular sensitivity was thought to be due to the degree of mitochondrial respiration in different cells, though; these results were in line with recent studies which established that N2A cells were the most affected by chemotherapy [15]. Therefore, endothelial cells were tested as a protective cell model within the neurogenic niche towards N2A cells from MTX.

Co-Culture of Different Components of Stem Cell Niche with Neural Cells to Determine if Any Components Protect Neural Stem Cells

Many studies have dealt with the cellular and molecular aspects of stem cells and some have confirmed that cellular interactions play a crucial role in stem cell differentiation and self-renewal by cell division. These interactions maintain the number and rate of division of stem cells as well as providing the appropriate environment by supplying growth factors and extracellular matrix molecules which ensure stem cell survival [26,27]. Endothelial cells are a prominent cellular component of the neurogenic niche and are known to secrete growth factors and extra cellular matrix including heparin sulfate glycosaminoglycan which stimulates neural stem cells [28]. These factors which include VEGF and BDNF are known to be directly involved in stimulating neurogenesis together with other signaling molecules [29]. In order to confirm the protective effect of brain endothelial cells against the effects of chemotherapy on N2A cells, these cells were co-cultured on a mono layer of endothelial cells prior to MTX treatment. The effect of chemotherapy was measured by quantifying N2A survival after 48hrs as compared to a control group of N2A cells treated

with MTX but not co-cultured with endothelial cells. The results showed that the number of N2A cells which survived MTX was significantly higher in the co-cultured wells.

As this could be a nonspecific effect of growing N2A cells on a cellular mono layer, the co-culture experiments were repeated with N2A cells co-cultured with a non-neuronal cell type, 3T3 fibroblasts. Co-culture with 3T3 cells showed no protection from MTX treatment at all indicating that protection is specific to endothelial cells.

These results build on studies that have found a close relationship between angiogenesis and neurogenesis. Many researchers have suggested that endothelial cells play a significant role in the proliferation of neural stem cells [30] and approximately 40% of proliferating neural stem cells in the SGZ are in contact with blood vessels. Endothelial cells have recently been shown in our lab to provide moderate protection to N2A cells from the chemotherapy agent 5-FU (Entedhar personal communication).

A lot of studies have investigated the deleterious effect of chemotherapy on cognition, but up until know there is little information about the possible mechanisms behind this and how some neural stem cells are able to continue to proliferate after chemotherapy treatment. The present project has developed a co culture system to examine the interactions between brain endothelial cells and neural cells. It is possible to hypothesize that the protective nature of endothelial cells is due to their production of growth factors, one of the most significant being Vascular Endothelial Growth Factor (VEGF). This is mainly secreted by endothelial cells in response to hypoxia and is involved in stimulating angiogenesis by activation of VEGF receptors. These are initiating a receptor tyrosine kinase signaling pathway which is important in vascular development [31]. It has been suggested that VEGF acts as a neurotropic protein and it has the ability to produce a neurogenic effect on neural progenitor cells [32]. This indicates that this factor may be associated with both angiogenesis and neurogenesis and provides a possible mechanism to explain our results.

In the absence of endothelial cells the number of N2A cells was decreased and that might be caused by a lack of VEGF which may reduce neural cellular differentiation, proliferation and self-renewal [27]. Another possible explanation of this protection is the ability of endothelial cells to produce many soluble cytokines and chemokines which are thought to influence cellular interactions and might be essential for the interactions between neurogenesis and angiogenesis. Wilhelmsson and his group have suggested that cell to cell contact is important for this interaction. This may be via the notch signaling pathway which uses cell membrane bound receptors and ligands. It is known that notch signaling is used by both endothelial and neural stem cells and so may be involved in the control of endothelial cells on N2A cells [33]. Notch signaling plays an important role in the proliferation, maturation and survival of neural stem cells [34].

A further possible explanation for the protection provided by niche cells could be due to the activation of the PTEN pathway which enables neural stem cells associated with blood vessels to be more resistant to chemotherapy. PTEN is a phosphatase which acts as a tumor suppressor gene that is involved in the pathophysiology of many malignancies like head and neck tumors and it is well known to play a significant role in brain development during early embryonic life [35]. *In vivo* studies have shown that PTEN knockout mice suffer from cognitive decline due to their inability to regulate neural stem cell proliferation and development [36]. Moreover, PTEN, P53 double knockout mice exhibit a reduction in neural stem cell differentiation and self -renewal due to the effect of elevated myc gene expression [37]. Thus PTEN is considered as a key factor in the development of neural stem cells and its expression may be one of the mechanisms related to endothelial cells induced neurogenesis.

CONCLUSION

The effect of brain endothelial cells on neurogenesis should be investigated more. The mechanism by which these cells provide cellular protection from MTX is not clearly understood and these interactions may be important in other aspects of cancer cell biology and response to chemotherapy. As indicated above a number of possible mechanisms can be suggested for these effects and which include direct cell to cell contact, activation of growth factors or cell signaling pathway. As many of these can be selectively blocked it should be possible to understand how this protection operates and if neural stem cells can be selectively protected, this would bring benefit to many patients treated with chemotherapy and finally reduce the symptoms of chemobrain among cancer survivors.

DECLARATIONS

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

[1] Swierkot, Jerzy, and Jacek Szechinski. "Methotrexate in rheumatoid arthritis." *Pharmacological Reports,* Vol. 58, No. 473, 2006, pp, 473-92.

[2] Shen, Sarah, et al. "The use of methotrexate in dermatology: A review." *Australasian Journal of Dermatology,* Vol. 53, No. 1, 2012, pp. 1-18.

[3] Seigers, Riejanne, et al. "Long-lasting suppression of hippocampal cell proliferation and impaired cognitive performance by methotrexate in the rat." *Behavioural Brain Research,* Vol. 186, No. 2, 2008, pp. 168-75.

[4] Wernick, Richard, and David L. Smith. "Central nervous system toxicity associated with weekly low-dose methotrexate treatment." *Arthritis and Rheumatism: Official Journal of the American College of Rheumatology,* Vol. 32, No. 6, 1989, 770-75.

[5] Aplin, C. G., and R. Russell-Jones. "Acute dysarthria induced by low dose methotrexate therapy in a patient with erythrodermic cutaneous T-cell lymphoma: An unusual manifestation of neurotoxicity." *Clinical and Experimental Dermatology,* Vol. 24, No. 1, 1999, pp. 23-24.

[6] Renard, Dimitri, et al. "Reversible posterior leucoencephalopathy during oral treatment with methotrexate." *Journal of Neurology,* Vol. 251, No. 2, 2004, pp. 226-28.

[7] Martino, Roy L., et al. "Transient neurologic dysfunction following moderate-dose methotrexate for undifferentiated lymphoma." *Cancer,* Vol. 54, No. 9, 1984, pp. 2003-05.

[8] Walker, Russell W., et al. "Transient cerebral dysfunction secondary to high-dose methotrexate." *Journal of Clinical Oncology,* Vol. 4, No. 12, 1986, pp. 1845-50.

[9] Haykin, M. E., et al. "Diffusion-weighted MRI correlates of subacute methotrexate-related neurotoxicity." *Journal of Neuro-Oncology,* Vol. 76, No. 2, 2006, pp. 153-57.

[10] Deangelis L. M., and Posner J. B. *Neurologic complications of cancer.* New York: Oxford University Press, 2009.

[11] Schagen, S. B., and J. Vardy. "Cognitive dysfunction in people with cancer." *The Lancet Oncology,* Vol. 8, No. 10, 2007, pp. 852-53.

[12] Winocur, Gordon, et al. "The effects of the anti-cancer drugs, methotrexate and 5-fluorouracil, on cognitive function in mice." *Pharmacology Biochemistry and Behavior,* Vol. 85, No. 1, 2006, pp. 66-75.

[13] Gandal, Michael J., et al. "A novel electrophysiological model of chemotherapy-induced cognitive impairments in mice." *Neuroscience,* Vol. 157, No. 1, 2008, pp. 95-104.

[14] Garthe, Alexander, Joachim Behr, and Gerd Kempermann. "Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies." *PloS One,* Vol. 4, No. 5, 2009, p. e5464.

[15] Han, Ruolan, et al. "Systemic 5-fluorouracil treatment causes a syndrome of delayed myelin destruction in the central nervous system." *Journal of Biology,* Vol. 7, No. 4, 2008, p. 12.

[16] Koppelmans, Vincent, et al. "Global and focal brain volume in long-term breast cancer survivors exposed to adjuvant chemotherapy." *Breast Cancer Research and Treatment,* Vol. 132, No. 3, 2012, pp. 1099-106.

[17] ELBeltagy, Maha, et al. "The effect of 5-fluorouracil on the long term survival and proliferation of cells in the rat hippocampus." *Brain Research Bulletin,* Vol. 88, No. 5, 2012, pp. 514-18.

[18] Lyons, Laura, et al. "The effects of cyclophosphamide on hippocampal cell proliferation and spatial working memory in rat." *PloS One,* Vol. 6, No. 6, 2011, p. e21445.

[19] Dietrich, Joerg, et al. "CNS progenitor cells and oligodendrocytes are targets of chemotherapeutic agents *in vitro* and *in vivo*." *Journal of Biology,* Vol. 5, No. 7, 2006, p. 22.

[20] Kim, Sun-Jin, et al. "Astrocytes upregulate survival genes in tumor cells and induce protection from chemotherapy." *Neoplasia,* Vol. 13, No. 3, 2011, pp. 286-98.

[21] Li, Na, et al. "D-galactose induces necroptotic cell death in neuroblastoma cell lines." *Journal of Cellular Biochemistry,* Vol. 112, No. 12, 2011, pp. 3834-44.

[22] Stins, Monique F., Floyd Gilles, and Kwang Sik Kim. "Selective expression of adhesion molecules on human brain microvascular endothelial cells." *Journal of Neuroimmunology,* Vol. 76, No. 1, 1997, pp. 81-90.

[23] Takeuchi, Takamasa, et al. "Characterization of mouse 3T3-Swiss albino cells available in Japan: Necessity of quality control when used as feeders." *Japanese Journal of Infectious Diseases,* Vol. 61, No. 1, 2008, p. 9.

[24] Kimmick G. G., et al. "The feasability of classical cychlophosphmide, methotrexate, 5-fluoruracil (CMF) for pre and post- menopausal node-positive breast cancer patients in a Belgian multicentric trial: A study of consistency in relative dose intensity and cumulative doses across institutions" *Annals of Oncology,* Vol. 13, No. 3, 2008, pp. 416-21.

[25] Cwikiel, M., et al. "The influence of 5-fluorouracil and methotrexate on vascular endothelium. An experimental study using endothelial cells in the culture." *Annals of Oncology,* Vol. 7, No. 7, 1996, pp. 731-37.

[26] Fuchs, Elaine, Tudorita Tumbar, and Geraldine Guasch. "Socializing with the neighbors: Stem cells and their niche." *Cell,* Vol. 116, No. 6, 2004, pp. 769-78.

[27] Jiao, Jianwei, and Dong Feng Chen. "Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals." *Stem Cells,* Vol. 26, No. 5, 2008, pp. 1221-30.

[28] Mercier, Frederic, John T. Kitasako, and Glenn I. Hatton. "Anatomy of the brain neurogenic zones revisited: Fractones and the fibroblast/macrophage network." *Journal of Comparative Neurology,* Vol. 451, No. 2, 2002, pp. 170-88.

[29] Mabie, Peter C., Mark F. Mehler, and John A. Kessler. "Multiple roles of bone morphogenetic protein signaling in the regulation of cortical cell number and phenotype." *Journal of Neuroscience,* Vol. 19, No. 16, 1999, pp. 7077-88.

[30] Shen, Qin, et al. "Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells." *Science,* Vol. 304, No. 5675, 2004, pp. 1338-40.

[31] Xie, Keping, et al. "Constitutive and inducible expression and regulation of vascular endothelial growth factor." *Cytokine and Growth Factor Reviews,* Vol. 15, No. 5, 2004, pp. 297-324.

[32] Jin, Kunlin, et al. "Vascular endothelial growth factor (VEGF) stimulates neurogenesis *in vitro* and *in vivo*." *Proceedings of the National Academy of Sciences,* Vol. 99, No. 18, 2002, pp. 11946-50.

[33] Wilhelmsson, Ulrika, et al. "Astrocytes negatively regulate neurogenesis through the Jagged1-mediated Notch pathway." *Stem Cells,* Vol. 30, No. 10, 2012, pp. 2320-29.

[34] Carlson, Morgan E., and Irina M. Conboy. "Regulating the Notch pathway in embryonic, adult and old stem cells." *Current Opinion in Pharmacology,* Vol. 7, No. 3, 2007, pp. 303-09.

[35] Backman, Stéphanie A., et al. "Deletion of Pten in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease." *Nature Genetics,* Vol. 29, No. 4, 2001, pp. 396-403.

[36] Groszer, Matthias, et al. "Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene *in vivo*." *Science,* Vol. 294, No. 5549, 2001, pp. 2186-89.

[37] Zheng, Hongwu, et al. "p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation." *Nature,* Vol. 455, No. 7216, 2008, pp. 1129-33.