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SCREENING THE EFFECT OF VASICINE IN MULTIPLE SCLEROSIS USING HUMAN TISSUE CHIP MODEL

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ABSTRACT: From time immemorial pharmacologists and researchers have always been in search for novel drug screening methods. One such unique technique that is gripping the world by its leash is "human tissue chip technology". This study explores the creation of Multiple Sclerosis (MS) using human tissue chip and assessing the ability of Vasicine a quinazoline alkaloid derived from *Adhatoda Justiciae* plant in reversing the inflammatory pathology of multiple sclerosis. 10 ml of human peripheral blood was withdrawn using a sterile syringe from a healthy donor. The blood was centrifuged to obtain mesenchymal stem cells (MSC). Then the MSCs were transferred with Dulbecco's Modified Eagle's medium (DMEM). After 48 h incubation, the MSCs were transferred into four flasks with growth factors and were seeded into tissue chip for differentiation into neuronal cells, cardiomyocytes, hepatocytes, and skeletal muscle cells. The respective cells were dislodged from tissue chip for histopathological evaluation and differentiation confirmation. Lipopolysaccharide (LPS) was added to these cells, to mimic MS inflammation and was confined to histo-pathological assessment. 9 biomarkers specific for Multiple sclerosis was assessed using RTPCR and gel electrophoresis pre and post Vasicine treatment. After 48 h inflamed neuronal cells reverted 80% normal cell morphology with Vasicine treatment. These cells exhibited only 20% remnant inflammation. Hence Vasicine can be further explored as a prospective molecule to treat Multiple sclerosis and this tissue chip technique can be used effectively as a single platform for culturing four different types of cells simultaneously. Thus various toxicological and disease conditions can be assessed using the tissue chip.

INTRODUCTION: Multiple Sclerosis is an autoimmune demyelinating disease of the central nervous system.

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It has 4 variants out of which relapsing remitting type is the most common. It affects usually young adults and most of the time creeps silently into the nervous system.

The prevalence of multiple sclerosis in Asia and South America is estimated to be <5 per 100 000 [1]. Being a complex disease, it is believed to affect women more commonly then men. There are certain states of the human body which may facilitate this disease condition, such as a pre-

existing immunosuppressed state of an individual, pregnancy, presence of auto-antibodies directed towards myelin antigens, certain level of genetic predisposition and environmental situations also contribute to this complex disease. There is no requirement of pre-existing inflamed central nervous system pathologies for MS to settle in rather it can affect an apparently normal brain too. The degeneration of the axons attributes to the chronic demyelination that occurs over the disease course [1]. As a part of MS, cognition decline is a commonly stated complaint by individuals affected by this disease. Pertaining to cognition, it more likely affects memory, attention verbal fluency *etc*. This is believed to occur due to the destruction of the myelin sheath which in turn causes a deficiency in "memory storage pathway" of the brain [2]. Many worldwide trials are being carried out to prevent the disease progression and improve cognition in individuals affected with Multiple Sclerosis.

Cognition will be enhanced if the inflammatory disease advancement is controlled. For this to occur many new drugs are being searched extensively by various experimental methods. Arbitrary methods for any drug discovery nowadays includes *in-silico* drug designing, followed by assays involving various diseased cell lines and finally tested in animals. However, even after successful preclinical studies, there have been many drugs that have failed to prove exuberance in many human studies. The failure for the drug to reach its successful "finish line" could be due to unavailability of IND testing methods in humans without their physical presence and them being left unharmed at any cost.

However as a light at the end of the tunnel " tissue chip" screening method is now becoming popular in which human stem cells maybe differentiated into various "units of organs" by adding growth factors and they may also be experimentally induced by disease conditions and then be monitored for their response to investigational molecules. This screening method will give a solid preliminary idea to a researcher about the capability of a drug to revert various disease conditions in human "organs" [3]. Thus this serves as a stepping stone to an era of clinical experiments which may not require human subjects as a whole but few millilitres of their peripheral blood itself would suffice to conduct toxicity studies, disease modifying drugs actions and so on. As a whole tissue chip screening method will drastically reduce the need for animal research and may take the investigational new drug to reach a stage just prior to approval of the drug directly. Precision and accurate human physiological correlation can be achieved without any hassle such as longer experimental durations, and more expenditure when done in rats [4].

In context to the blood used for carrying out human tissue chip studies, usually cord blood, or direct donor cells are used for attaining stem cells which are then made to differentiate into required cells. However, there have been recent studies which used mesenchymal cells derived from peripheral blood which exhibited similar chondrogenic features as that of the cells derived from bone marrow [5]. With this potentiality of the MSC derived from peripheral blood, in this current study, they have been differentiated into neuronal, cardio myocytes, hepatocytes and skeletal muscle cells with the addition of respective growth factors.

Moreover, the tissue chip used in this present study varies from the technologically advanced tissue chips which are more bulky, requiring osmotic pumps. The material that is used for the tissue chip is a biopolymer. The dimensions of this chip are more customized than the conventional chip. It measures 15 mm in length, 5 mm breadth and 3 mm in height. After much screening, this tissue chip has been used for this study. The fact that this tissue chip does not require any osmotic pumps but instead can directly be lodged into the culture media, makes it a smarter technology to screen drugs or conduct toxicity studies. The chances for contamination are also lower when compare to the conventional tissue chips working with osmotic pumps.

In this current study, Vasicine which is derived from *Adhatoda justiciae* plant was explored for its multiple sclerosis reversal properties by being added to the tissue chip organ models. As such Vasicine is a well-known quinazoline alkaloid which has extensive application as bronchodilators in respiratory conditions [6]. However, its role in neurological and neurodegenerative disorders has been scarcely studied. There has been a literature suggestive of Vasicine possessing Acetyl-

cholinesterase and Butyrylcholinesterase weak inhibitory activity [7]. However, its role in multiple sclerosis or other neurodegenerative conditions has not been well studied. Now in this study, Vasicine's role in Multiple Sclerosis disease regression is investigated.

METHODS:

Preparation of Tissue Chip and Differentiation into Respective Cell Types: 10 ml of human blood was withdrawn using a sterile syringe from a healthy donor. Institutional Ethical clearance was obtained (IEC-NI/18/JAN/63/13). Density gradient centrifugation was done. The blood was allowed to stand for 10 min and the top layer of the red blood cells was pipetted to a centrifuge tube. This supernatant was centrifuged at around 9000 rpm for 10 min and cells were transferred to 10 ml of RPMI media and incubated at 37º in 5% CO_2 incubator for 48 h to promote adhesion of mesenchymal stem cells to the bottom layer of the flask. After 48 h the supernatant was discarded and the mesenchymal stem cells were mechanically dislodged using cell dislodger and gentle shaking. Mesenchymal stem cells were spun at 12,000 rpm for 15 min at 25 ºC and seeded into fresh DMEM without glucose and Fetal Bovine Serum and incubated at 37 ºC incubator with 5% CO_2 for 48 h. The final concentration of cells was 1.5×10^5 Cells per ml. After 48 h, mesenchymal stem cells were transferred into 4 cell culture flasks with varying proportion of growth factors for differentiation to precursor cells at a final concentration of 10 nanomoles.

Post incubation of the induced mesenchymal stem cells, they were transferred to the tissue chip with tryptophan 0.024 and Leucine 0.130 cell culture mix. Cells were centrifuged at 12,000 rpm for 15 min at 25 ºC and the pellet was re-suspended in 100ul DMEM. 10ul of the pellet of each of the precursor cells at 3×10^5 Cells per ml were injected using a syringe into each of the matrices to allow differentiation into neuronal cells, cardio myocytes, hepatocytes and skeletal muscle cells. This method of culturing four different types of cells in a single tissue chip platform has been patented (Indian Patent Application number 201741001970). Leukocytes from the supernatant were added to the cell culture media to act as similar to those in the blood. After incubation for 96 h at 37º in 5% CO_2 incubator the cells the tissue chip was ready for screening.

Induction of Multiple Sclerosis in Tissue Chip Model: 10 µl of bacterial lipopolysaccharide along with hydrophobic neuronal membrane proteins equalling to a final concentration of 5 nanomoles per ml was added to the tissue chip matrix containing neuronal cells to induce inflammation. Hydrophobic neuronal membrane proteins were extracted from neuronal precursor cells. Development of inflammation was noted with marked increased in leukocytes to 12×10^5 Cells per ml in 48 h with inflammatory cells also binding to the neuronal cells. Marked degeneration of neuronal cells with severe inflammation was observed in the matrix for neuronal cells.

Expression of Biomarkers: Expression of MS biomarkers was confirmed by RT- PCR and gel electrophoresis. A total of 9 Biomarkers including Nogo-A, a diagnostic biomarker, CXCL 12, Fetuin-A were assessed. The results are given in **Fig. 1**.

Screening for Effect of Vasicine: Vasicine was added at a final concentration of 0.6 Nano gram per ml to Multiple Sclerosis induced tissue chip and incubated for 48 h before histopathological evaluation. The cells were dislodged from the tissue chip after 48 h, followed by smear preparation using eosin, haematoxylin stain, finally observed under a light microscope for histo-pathological assessment.

RESULTS: A total of 9 biomarkers were screened quantitatively, to measure the effect of the reversal of Multiple sclerosis induced neuronal cells to normalcy after due treatment with Vasicine. It was observed in general that there was a decrease in expression of the biomarkers post treatment with Vasicine. The expression of the concerned biomarkers in normal is given in **Fig. 1** below.

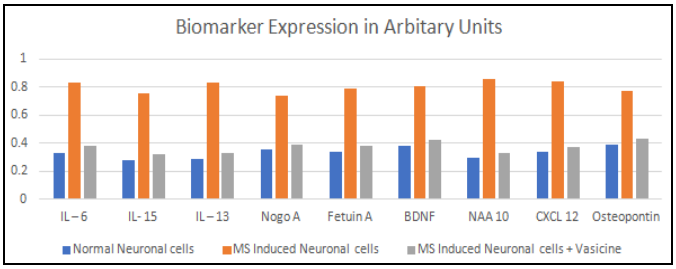
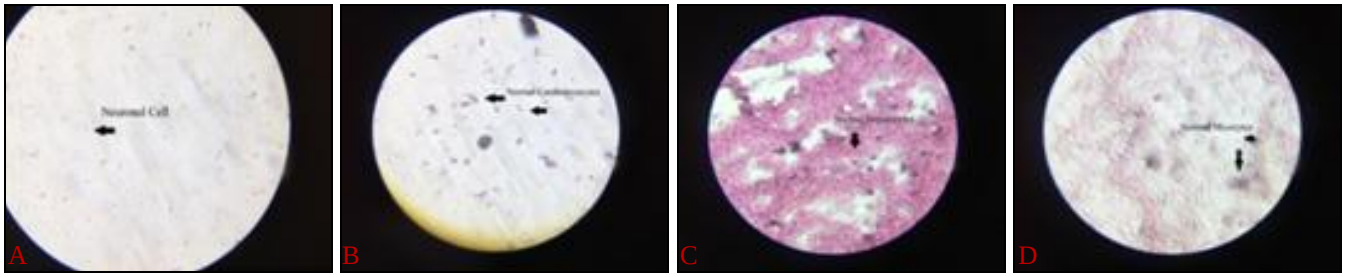


FIG. 1: HISTOGRAM OF MS BIOMARKERS EXPRESSED BEFORE AND AFTER TREATMENT WITH VASICINE

Following this, the histopathological assessment of multiple sclerosis induced neuronal cells before and after treatment of Vasicine exhibited 80% of normal neuronal cells and the number of leukocytes also decreased from 12×10^5 cells per ml to 6×10^5 cells per ml. The histopathology pictures of normal differentiated mesenchymal cells into neuronal cells, cardio myocytes, hepatocytes, and myocytes are given in **Fig. 2 A, B, C, D**.



NEURONAL CELLS CARDIOMYOCYTES HEPATOCYTES MYOCYTES

FIG. 2: HISTOPATHOLOGY OF DIFFERENTIATED MESENCHYMAL CELLS INTO NEURONAL CELLS, CARDIOMYOCYTES, HEPATOCYTES AND MYOCYTES

Histopathology pictures of LPS induced inflamed neuronal cells are given in **Fig. 3A, B**. Post Vasicine treatment inflamed neuronal cells exhibiting normalcy is depicted in histopathology pictures **Fig. 4A, B**. The tissue chip used in this study is given in **Fig. 5A**.

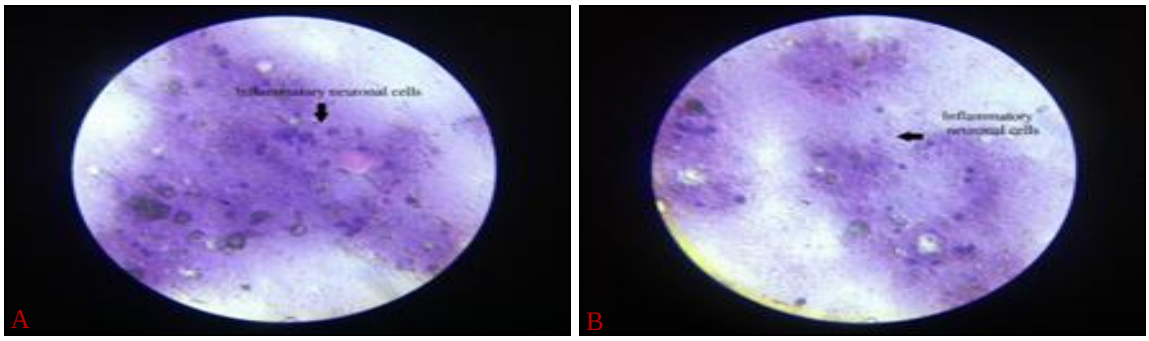


FIG. 3: HISTOPATHOLOGY OF LPS INDUCED INFLAMMATORY NEURONAL CELLS

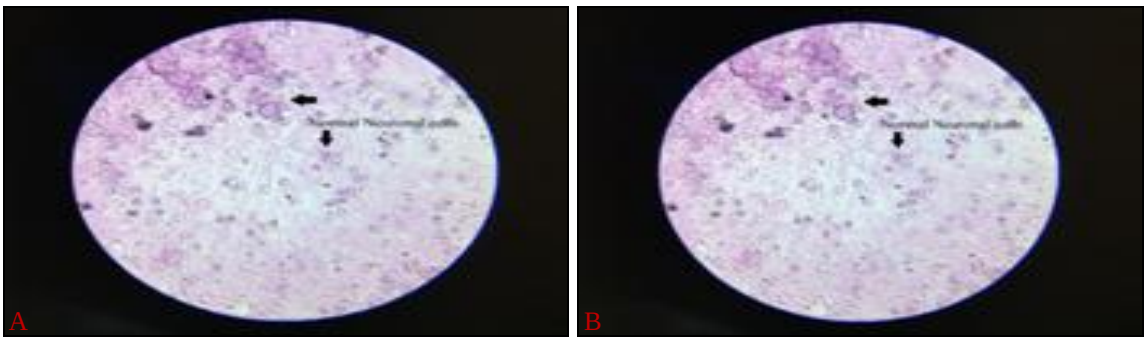


FIG. 4: HISTOPATHOLOGY OF NORMAL NEURONAL CELLS POST VASICINE TREATMENT

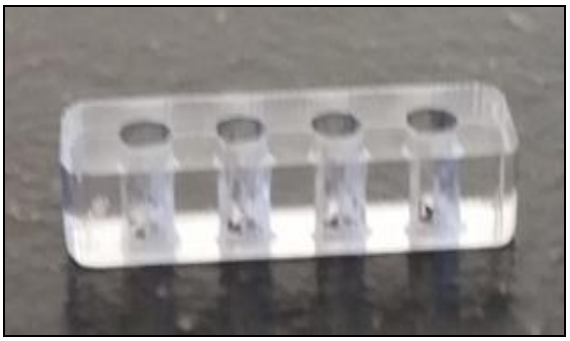


FIG. 5: TISSUE CHIP USED IN THIS STUDY

DISCUSSION: It is clearly evident from these results that Vasicine has potent activity in reverting inflammatory neuronal cells up to 80% to normalcy in MS induced tissue chip model. The basic architecture of the cell is reverted to normal post Vasicine treatment. This is observed in the histopathology images. To add on Vasicine has also shown its effect in decreasing the number of leukocytes from 12×10^5 cells per ml to 6×10^5 cells per ml which correlates to the point that the inflammation is decreasing.

However, whether Vasicine has the ability to suppress T lymphocytes, in particular, require further studies as suppressing the lymphocytic proliferation or activation, which is recently attributed to the causation of demyelination in Multiple sclerosis subjects may prove beneficial to the mankind [8]. This study also provides a clear insight into using the tissue chip as a potential platform to "grow" various types of cells in a single 3D matrix model at the same point in time. This not only decreases the period required to grow various types of cells at the same time but also serves as a novel technique in microfluidic cell culture system [9] without requiring any sophisticated systems.

Thus, tissue chip platform will also be useful in carrying out preliminary toxicological studies of a drug directly using animal tissues or differentiated human cells replacing whole animal toxicity experiments. Instead of routinely using stem cells obtained from cord blood to differentiate into various other cells, using the mesenchymal cells present in the peripheral human blood for differentiating into other types of cells seems to be a less cumbersome and an easy process.

Moreover, the biomarker profiling also confirmed the decrease in the concerned biomarkers of multiple sclerosis post Vasicine treatment when compared to the biomarkers expressed after inflammatory induction with LPS in neuronal cells. The reduced biomarker expression after Vasicine treatment is close to the normal value range of biomarker expression in normal neuronal cells. These results are not only useful to arrive at a preliminary conclusion that it can serve as a lead molecule itself in multiple sclerosis treatments but indirectly it may also alleviate cognitive dysfunctions and deficiencies that may subtly develop in MS individuals. But for this to be strappingly confirmed further studies will be steered especially establishing a correlation between improvement of MS with Vasicine treatment and subsequent cognition improvement in animal models of MS. Moreover additional studies will also be conducted to explore the molecular level mechanism with which Vasicine may be able to recuperate cognition mutilation in MS. As Vasicine also possesses antioxidant properties, [10] it may broaden the probability of Vasicine which is a plant derived bioactive molecule, in aiding cognition enhancement [11].

CONCLUSION: Vasicine, from this preliminary study, proves to have potential reversal effects on neuronal inflammation in a Multiple sclerosis human tissue chip model for the first time (to the best of our knowledge). Hence, it can be to a certain extent agreed that Vasicine may serve as a very promising molecule for the treatment of Multiple sclerosis, in solidly decreasing the disease progression. In addition, this current study has also given a hopeful outcome that human tissue chip drug screening method may serve as a preliminary method to obtain on how a drug or molecule may establish its effect on human cells. Thus the time and resources spent on assessing a novel molecule for its possible effects in humans can be predetermined at an earlier stage using this technique. However, further studies are required to confirm the firm action of Vasicine in decreasing or halting the disease progression in Multiple sclerosis.

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CONFLICT OF INTEREST: Authors declare there is no conflict of interest.

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