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SCREENING OF HOMOEOPATHIC FORMULATION FOR ENHANCEMENT OF NEUROGENIC DIFFERENTIATION OF UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS (UCMSCS)

Himanshu Shekhar[1], Parth Aphale[2*] and Dharmendra Sharma[3]

[1],[2]Department of Homoeopathic Pharmacy, [3]Department of Forensic Medicine & Toxicology, Dr. D.Y. Patil Homoeopathic Medical College & Research Centre, Dr. D.Y. Patil Vidyapeeth (Deemed to be University), Pimpri, Pune, Maharashtra (India)
*****e-mail: *parth.aphale@dpu.edu.in*

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ABSTRACT

Mesenchymal stem cells are multipotent cells derived from various tissues, with potential to differentiate into various cell types. They possess immuno-modulatory properties and hold promise for therapeutic applications in neuro-degenerative diseases and autoimmune disorders. Homeopathic remedies like *Plumbum metallicum (Plumb-met), Zincum metallicum (Zinc-met),* and *Agaricus muscarius (Agari-musc),* are used to treat neuralgic pains and central nervous system disorders, However, the precise pathophysiological mechanisms underlying their actions remain unestablished. This study was aimed to assess the efficacy of homoeopathic medicines *Plumb-met, Zinc-met,* and *Agari-musc* on umblical cord derived mesenchymal stem cells (UCMSCs) and assess their cytotoxicity and potential in neuronal differentiation evident by highest morphological transition into neuronal-like cells evident by exhibition of fine neurite extensions. UCMSCs were isolated and cultured. MTT assays were performed to evaluate the cytotoxicity of *Plumb-met, Zinc-met,* and *Agari-musc* at various potencies (6C, 12C, 30C, 200C, and 1M) and concentrations (2, 1, and 0.5%) over a 9-day induction period. Neuronal differentiation was induced, and the resulting neuronal cells were compared to a positive control. MTT assay showed the mean percent cytotoxicity was -180.3 ± 52.28 for *Plumb-met,* -201.4 ± 72.29 for *Zinc-met,* -174.1 ± 69.18 for *Agar-musc* (mean ± SEM; n = 5). There was no cytotoxicity due to 0.5% concentration of *Plumb-met* 6C, *Zinc-met* 6C and *Agari-musc* 6C when correlated with control. Notably, *Zinc-met* 6C @ 1, 0.5, and 2% exhibited a significant increase in neuronal cells and extensive synapse formation. *Plumb-met* 6C and *Agari-musc* 6C also demonstrated neuronal differentiation and synapse formation, albeit to a lesser extent compared to *Zinc-met* in the 6C potency. This study indicated that *Zinc-met* (6C) has the potential to enhance neuronal differentiation in UCMSCs.

Keywords: Homeopathic treatments, mesenchymal stem cells, umbilical cord derived mesenchymal stem cells (UCMSCs), neurogenic differentiation

INTRODUCTION

Mesenchymal stem cells (MSCs) are versatile type of multipotent cells that can be derived from various tissues, including bone marrow, umbilical cord, and adipose tissue. These cells show the ability to differentiate into a wide range of cell types, including osteocytes, chondrocytes, adipocytes, cardiomyocytes, hepatocytes, and neural cells (Datta *et al.,* 2023). These MSCs also have immuno-modulatory activity, so MSCs can be used in various cell based therapeutic option for various

disorders like Alzheimer's and Parkinson diseases. Moreover, MSCs has been reported to be used for metabolic and degenerative disorders such as myocardial infarction, graft versus host disease, diabetes, liver cirrhosis, spinal cord injury, osteoporosis, and osteoarthritis (Sanap *et al.,* 2021).

Bone marrow emerges as a primary reservoiur of MSCs, and constitutes a prominent source in both experimental and clinical studies targeting various disorders. However, it is imperative to know the constraints associated with bone marrow-derived MSCs, particularly diminished yield and compromised differentiation potential with advancing age (Zhou *et al.,* 2008; Wagner *et al.,* 2009; Stolzing *et al.,* 2023). The alternative sources of MSCs have gained prominence, with umbilical cord standing out as a promising and easily accessible source due to its non-invasive tissue extraction, minimal environmental impact, lack of age-related concerns, and absence of ethical concerns.

The theoretical framework of neurogenesis is based on the ability of stem or progenitor cells to differentiate into neurons. By applying this framework to mesenchymal cells, we aimed to assess their phenotypic transformation into neuronal-like cells when exposed to homeopathic remedies. Additionally, the study incorporated established methods for assessing neuronal differentiation and phenotypic changes, such as morphological alterations and proliferation status, to comprehensively analyse the effects of *Plumb-met, Zinc-met,* and *Agari-musc* on mesenchymal cells. Based on anecdotal literature homoeopathic preparations of *Plumb-met, Zinc-met,* and *Agari-musc* are therapeutically useful in treating the localized neuralgic pains and neuritis, management of central nervous system (CNS) diseases, management of various forms of neuralgia and spasmodic affections (Boericke, 2000). The said homoeopathic remedies *Plumb-met, Zinc-met,* and *Agari-musc* are widely used in treating neuralgia, neuritis, CNS disorders and spasmodic affections but it is noteworthy that the underlying pathophysiological mechanisms of action of these homeopathic remedies on human body remain unestablished. Therefore, in this study the UCMSCs were cultured and treated with different centesimal potency of homoeopathic remedies *Plumb-met, Zinc-met,* and *Agari-musc* to assess their patho-physiological action.

There is growing interest in alternative complementary medicine, including homeopathy, for potential use in neurogenesis, especially against neurodegenerative diseases where novel and safe therapeutic approaches are needed. Studies on the effects of homeopathic medicines on neuronal differentiation is justified due to their long-standing use in clinical practice and the need for scientific scrutiny of their potential efficacy. The present study was aimed to assess the effect of homeopathic remedies on mesenchymal cell phenotypes during neuronal differentiation, contributing to the broader understanding of their mechanisms of action. The primary goal was to assess the potential neurogenic effects of homeopathic medicines *Plumb-met, Zinc-met,* and *Agari-musc* on mesenchymal cells undergoing neuronal differentiation. We aimed to understand whether these homeopathic remedies induce phenotypic changes in mesenchymal cells during neuronal differentiation and explore their potential as agents for promoting neurogenesis.

MATERIALS AND METHODS

The present study was conducted at Regenerative Medicine Laboratory, Dr. D.Y Patil Dental College, Pimpri, Pune (India) from September 2023 to March 2024. This laboratory is well equipped with all the facilities required for the isolation and culture of UCMSCs. All the studies were conducted in triplicate. The ethical approval for the present study was obtained from the Institutional Committee for Stem Cell Research vide reference No. IC-SCR/RM/50/22.

Procurement of homoeopathic medicine

Homoeopathic medicines (*Plumb-met, Zinc-met* and *Agari-musc*) in 6C, 12C, 30C, 200C, 1M were procured from GMP approved Homoeopathic Pharmaceutical Industry. In homeopathy, the potency of medicines such as *Plumb-met, Zinc-met,* and *Agari-musc* is indicated by the numbers followed by

letters "C" and "M" where "C" stands for centesimal (wherein the substance is diluted in the ratio of 1 part of medicinal substance to 99 parts of alcohol). For example, 6C means the substance was diluted 1:100 six times, 12C means it has been diluted 1:100 twelve times, 30C means it has been diluted 1:100 thirty times, and 200C means it has been diluted 1:100 two hundred times. The "M" stands for 1M = 1000C. These dilutions are believed to enhance the therapeutic effects of medicines in homeopathic practice.

Collection and culture of UCMSCs

The UCMSCs utilized in this study were sourced from Dr. D. Y. Patil Ayurved College & Hospital and cultured at Dr. D.Y. Patil Dental College & Hospital. The UCMSC culture protocol closely followed the established methodology (Datta *et al*., 2023). Specifically, the cells were initially seeded in knock-out Dulbecco's modified Eagle's medium (DMEM-KO; GibcoBRL), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.5% Penstrep. The cultures were then maintained in a controlled environment at 37°C with a 5% humidified CO_2 atmosphere. The culture medium was refreshed at regular intervals of every 3 days, ensuring optimal growth conditions. Sub-culturing of cells was performed when they reached approximately 85% confluence. Subsequently, these UCMSCs were employed for experimentation following confirmation of their identity through the verification of MSC markers. MTT assays were performed to evaluate the cytotoxicity of *Plumb-met*, *Zinc-met*, and *Agari-musc* at various potencies (6C, 12C, 30C, 200C, and 1M) and three concentrations (2, 1, and 0.5%) over a 9-day induction period.

Neuronal differentiation

The neuronal differentiation of UCMSCs into a neuronal cell type were carried out as per Trzaska *et al.* (2023). UCMSCs were subjected to trypsinization and subsequently plated in four-well chamber slides. The neuronal induction medium used consisted of neurobasal medium supplemented with 0.5% B27 supplement. Additionally, this medium was enriched with a cocktail comprising of 250 ng mL^{-1} sonic hedgehog (SHH), 100 ng mL^{-1} fibroblast growth factor 8, and 50 ng mL^{-1} basic fibroblast growth factor. The plated cells were then incubated at 37°C in an atmosphere containing 5% CO_2. During incubation, they were induced to undergo neurogenic differentiation and were subsequently maintained in neurobasal medium supplemented with 0.5% B27. For control group, a separate set of MSCs was cultivated in neurobasal medium with B27 supplement alone, devoid of the induction cocktail. The treatment groups comprised of i) Control group - MSCs, ii) MSCs culture in neurobasal medium, iii) MSCs culture in neurobasal medium + induction cocktail, iv) MSCs culture in neurobasal medium + induction cocktail + homoeopathic medicines with different potencies. *Plumb-met*, *Zinc-met* and *Agari-musc* were evaluated at potency of 6C, 12C, 30C, 200C and 1M level. *Plumbum metallicum*, derived from lead, is traditionally used in homeopathy to address neurological symptoms and disorders. *Zincum metallicum*, derived from zinc, is commonly used for its supposed effects on nervous system, particularly for conditions involving neuralgia and spasms. *Agaricus muscarius*, derived from agaric mushroom, is often used in homeopathy for its neurological effects, including tremors and convulsions. The concentrations evaluated were 0.5, 1.0 and 2.0%.

Experimental details

The experiment was designed to evaluate the effects of various concentrations of homeopathic medicines on neurogenic differentiation of UCMSCs. The concentrations evaluated were 0.5, 1.0 and 2.0%. Each treatment was replicated thrice to ensure the statistical reliability and accuracy of results. The parameters studied included cell viability and morphological changes. The cell viability and proliferation was assessed by using MTT or trypan blue exclusion assays. The morphological changes were noticed by observing the changes in cell morphology indicative of neuronal differentiation.

Statistical analysis

The cell viability and proliferation of UMSCs was expressed as mean ± standard deviation of the experimental data. Statistical differences among all groups were evaluated using one-way analysis of variance (ANOVA), with a significance threshold set at $P < 0.05$. The differentiation of UMSCs into

neuronal stem cells are determined by highest morphological transition into neuronal-like cells evident by exhibition of fine neurite extensions (Al-Maswary *et al.*, 2022).

RESULTS AND DISCUSSION

Morphology of UCMSCs

Microscopically, the Umbilical Cord-derived mesenchymal stem cell samples were observed as thin, elongated, and flattened cells with small cell bodies and slender processes. These observations were made using an Olympus biological microscope - trinocular Halogen (MX 21i) at a magnification of 10X (Fig. 1).

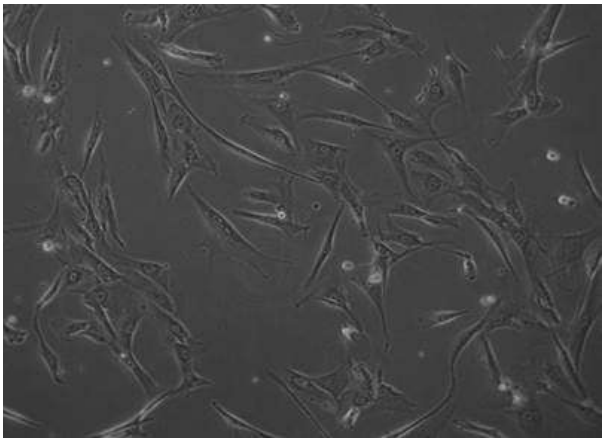

Fig. 1: Morphology of MSCs isolated from human umblical cord (magnification 10X)

The MTT assay was done to assess the toxic effect of *Plumb-met* 6C, *Zinc-met* 6C and *Agari-musc* 6C on MSCs using 0.5% concentration of *Plumb-met*, *Zinc-met* and *Agari-musc* for 24 h. MTT assay showed the mean percentage cytotoxicity was –180.3 ± 52.28 for *Plumb-met*, –201.4 ± 72.29 for *Zinc-met*, –174.1 ± 69.18 for *Agar-musc* (mean ± SEM; n = 5). MTT assay showed that there was no cytotoxicity due to 0.5% concentration of *Plumb-met* 6C, *Zinc-met* 6C and *Agari-musc* 6C when correlated with the control (Fig. 2).

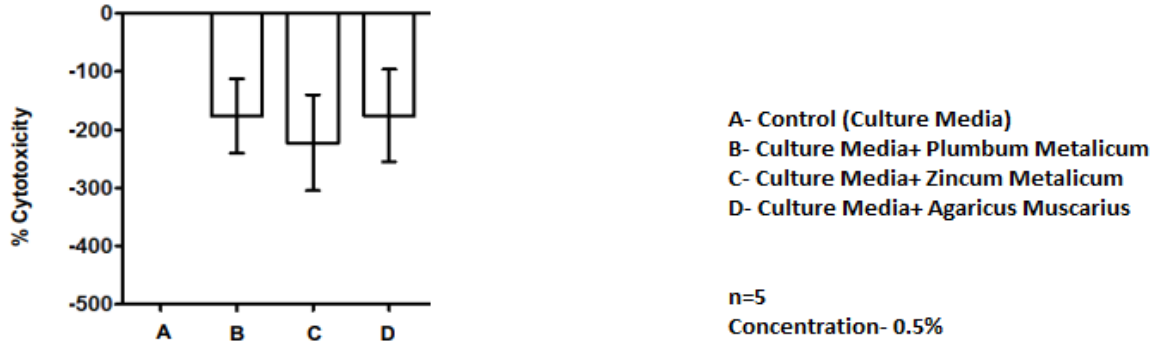

Fig. 2: MTT assay of UCMSCs treated with 0.5% concentration of *Plumb-met* 6C, *Zinc-met* 6C, and *Agari-musc* 6C (0.5% v/v)

Umbilical cord derived MSCs (UCMSCs) differentiated into neuronal cells in 9 days

Neuronal differentiation was induced in UCMSCs for 9 days for each potency of *Plumb-met*, *Zinc-met*, and *Agari-musc* in 6C, 12C, 30C, 200C, 1M using 2, 1, 0.5% concentration that was added to Neuronal induction medium with 0.5% B27 supplement. It also contained a cocktail of 250 ng mL^{-1} SHH, 100 ng mL^{-1} fibroblast growth factor 8 and 50 ng mL^{-1} basic fibroblast growth factor. As a control, MSC's were also grown with neurobasal medium and B27 supplement alone, without the induction cocktail. After 9 days, the UCMSCs of *Plumb-met*, *Zinc-met*, and *Agari-musc* in 6C in each concentration 2, 1, and 0.5% showed altered structure from spindle shaped cells to refracted cell bodies with long, thin processes, resembling distinct neuron-like morphology (Fig. 3-6). Interestingly, the group treated with homoeopathic remedy *Zinc-met* 6C showed neuron like morphology with synapse formation. However, the group treated with *Plumb-met*, *Zinc-met* and *Agari-musc* in 12C, 30C, 200C, 1M did not show any alteration of morphology of UCMSCs.

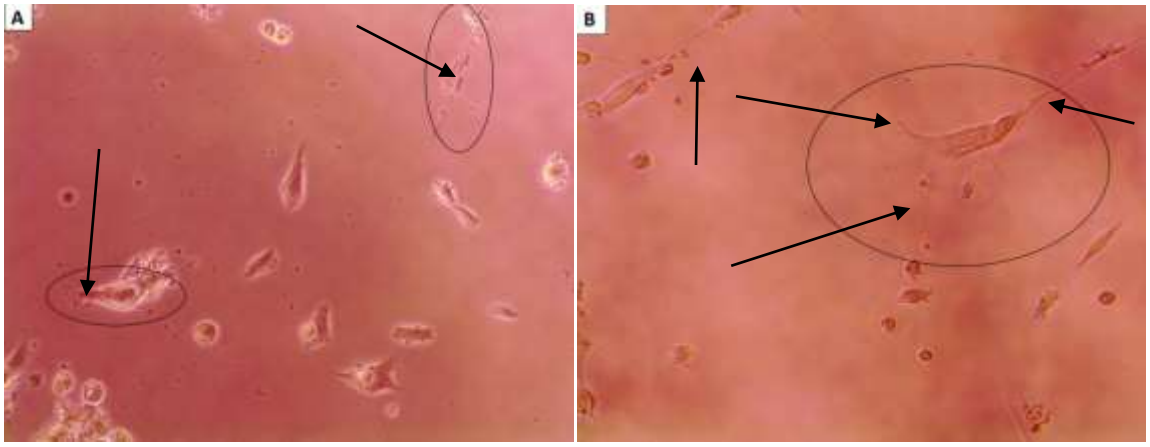

Fig. 3: A) Positive control (MSCs culture in neurobasal medium + induction cocktail) shows neurons with dendrites as well as synapse formation; B) Extensive synapse formation with other neurons is visible. Solid arrows indicate bipolar morphology or multipolar neuronal-like morphology

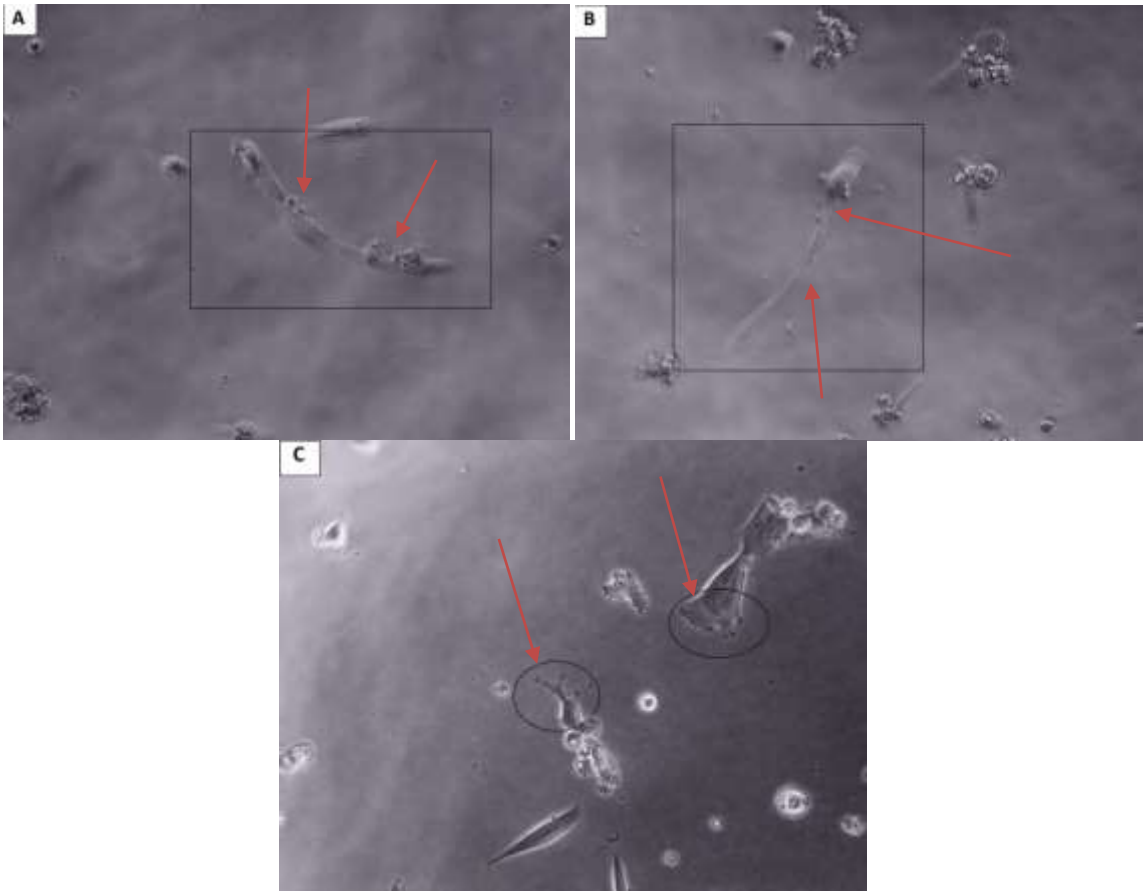

Fig. 4: 0.5% concentration A) Group treated with 0.5% *Agari-musc* shows synapse formation B) Group treated with 0.5% *Plumb-met* shows synapse formation; C) Group treated with 0.5% *Zinc-metal* shows Neurons with dendrites. Solid arrows indicate bipolar morphology or multipolar neuronal-like morphology

Fig. 3 shows the neuronal differentiation in positive control group which contained neurobasal medium plus induction cocktail. Positive control group clearly indicates neuron cells with dendritic processes. This group also shows the extensive synapses formation. Fig. 4 indicated the group treated

with 0.5% concentration of homoeopathic remedies *Plumb-met*, *Zinc-met* and *Agari-musc*. Image A was of group treated with 0.5% *Agari-musc* indicated synapse formation; Image B was of group treated with 0.5% *Plumb-met* showed synapse formation and image C was group treated with 0.5% *Zinc-met* showed neurons with dendrites. Fig. 5 indicated the group treated with 1% concentration of Homoeopathic remedies *viz*., *Plumb-met, Zinc-met,* and *Agari-musc*. Image A was of the group treated with 1% *Agari-musc* showed dendrites with synapse formation; image B of group treated with 1% *Plumb-met* showed nerve cells with dendrites and image C of group treated with 1% *Zinc-met* showed neurons with dendrites along with a long chain of synapses with other nerve cells. Fig. 6 indicated the group treated with 2% concentration of homoeopathic remedies *Plumb-met, Zinc-met,* and *Agari-musc*. Image A of the group treated with 2% *Agari-musc* showed synapse formation; image B of the group treated with 2% *Plumb-met* showed nerve cells forming synapses and image C of the group treated with 2% *Zinc-met* showed neurons with dendrites along with a synapses with other nerve cells.

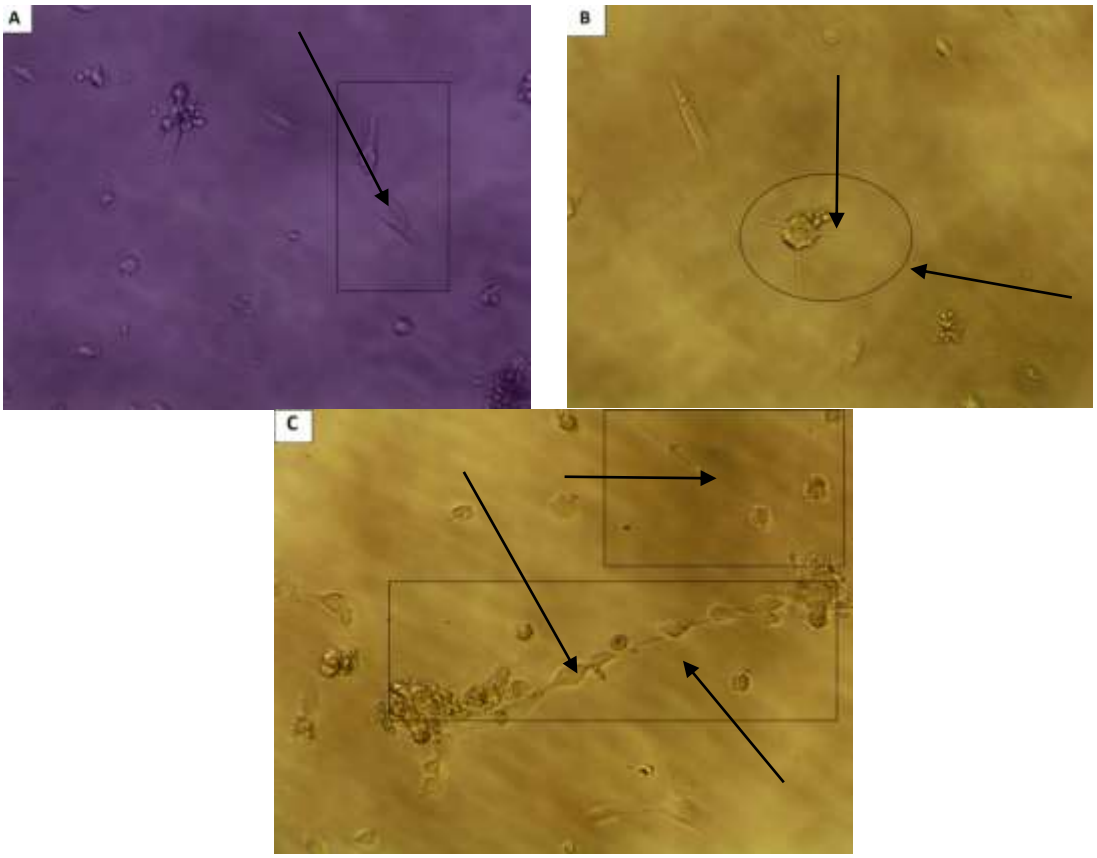

Fig. 5: 1% concentration; A: The group treated with 1% *Agari-musc* shows dendrites with synapse formation; B: the group treated with 1% *Plumb-met* shows nerve cells with dendrites; C: the group treated with 1% *Zinc-met* shows neurons with dendrites along with a long chain of synapses with other nerve cells. Solid arrows indicate bipolar morphology or multipolar neuronal-like morphology

In this study, it was observed that each group treated with 0.5, 1 and 2% concentration of homoeopathic remedy *Plumb-met, Zinc-met,* and *Agari-musc* in 6C potency induced neuronal differentiation as the stem cells were differentiated into neuronal cells after treatment. On comparison *Zinc-met* in 1, 0.5 and 2% concentration showed larger number of neuronal cells and extensive synapses was also observed. Other groups i.e. *Plumb- met* and *Agari-musc* also showed neuronal differentiation and synapses formation.

The human body has capability to repair and regenerate itself after any injury. However, there are many conditions in which human body is unable to repair and regenerate itself. The stems cells

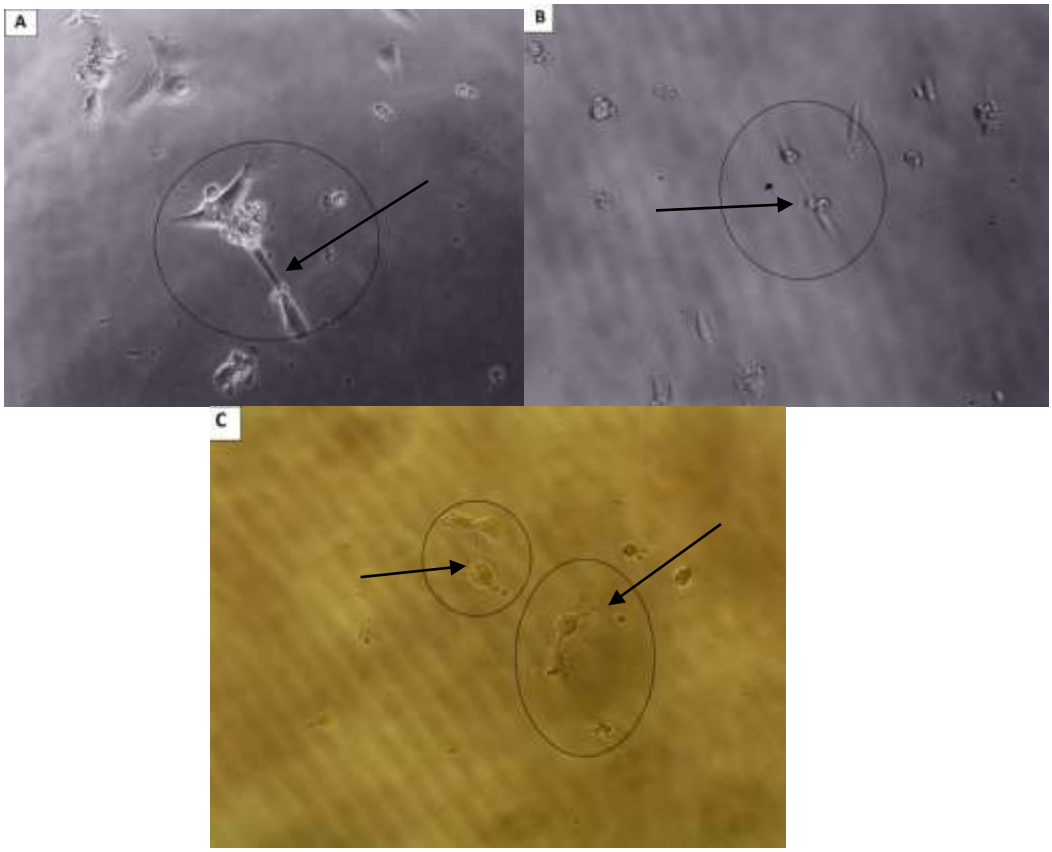

Fig. 6: 2% concentration; A: Group treated with 2% *Agari-musc* shows synapse formation; B: Group treated with 2% *Plumb-met* shows Nerve cells forming synapses; C: Group treated with 2% *Zinc-met* shows Neurons with dendrites along with a synapses with other nerve cells. Solid arrows indicate bipolar morphology or multipolar neuronal-like morphology

have immuno-modulatory activity, and therefore MSCs are used in various cell based therapeutic option in conditions like Alzheimer's and Parkinson disease and many more. These stem cells are present in every human being but in latent form. With any neurogenic inducers or any agents which can stimulate these stems cells which were in latent form, these can be made active and can show immuno-modulatory activity by releasing the biomarkers which can be extremely beneficial in various therapeutic options.

Plumb-met, *Zinc-met*, and *Agari-musc* are neurogenic inducers or agents derived from minerals (*Plumb-met* from lead, *Zinc-met* from zinc) and mushroom (*Agari-musc*). On the basis of anecdotal literature homoeopathic preparations of *Plumb-met*, *Zinc-met* and *Agari-musc* are therapeutically useful in treating the symptoms related to central nervous system like localized neuralgic pains and neuritis, management of diseases with brain and spinal symptoms, management of various forms of neuralgia and spasmodic affections (Boericke, 2000). In this study it was observed that each group treated with 0.5, 1 and 2% concentration of homoeopathic remedy *Plumb-met*, *Zinc-met*, and *Agari-musc* in 6C potency induced neuronal differentiation as the stem cells were differentiated into neuronal cells after the treatment. On comparison, *Zinc-met* in 1, 0.5 and 2% concentration showed a larger number of neuronal cells and extensive synapses. Other groups i.e. *Plumb-met* and *Agari-musc* also showed neuronal differentiation and synapses formation.

The MTT assay demonstrated that 6C potency of *Plumb-met*, *Zinc-met*, and *Agari-musc* were not cytotoxic to UCMSCs. Also, it was noted that 6C potency of *Plumb-met*, *Zinc-met*, and *Agari-musc* enhanced the proliferation of UCMSCs. UCMSCs were differentiated into neuronal cell type as described by Trzaska *et al*. (2023). The neuronal induction medium contains neurobasal medium with

0.5% B27 supplement. It also contains a cocktail of 250 ng mL^{-1} SHH, 100 ng mL^{-1} fibroblast growth factor 8 and 50 ng mL^{-1} basic fibroblast growth factor. Microscopic examination confirmed the differentiation of UCMSCs into neuronal cells in all the treated groups. Also, they resembled the morphology of neuronal cells (Datta *et al*., 2023).

Conclusion: Present study suggested that the homeopathic remedies *Plumb-met*, *Zinc-met*, and *Agari-musc* in 6C potency can promote the differentiation of UCMSCs into neuronal cells. Notably, *Zinc-met* 6C at 1, 0.5, and 2% concentrations demonstrated higher neuronal differentiation and synapse formation. Though *Plumb-met* 6C and *Agari-musc* 6C facilitated neuronal differentiation, their effects were less pronounced as compared to *Zinc-met* 6C. The exposure to neural induction medium resulted in distinct phenotypic changes in mesenchymal cells, including cytoplasmic retraction, spherical cell body formation, and cellular protrusions, indicating a departure from baseline MSC morphology (Krampera *et al.*, 2007). These findings highlight the potential of homeopathic remedies to influence UCMSC differentiation. However, further research is needed to confirm the nature of cytoplasmic extensions and to strengthen the evidence supporting the therapeutic potential of these remedies in neuronal differentiation.

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Ethical statement: The ethical approval for present work was procured from the Institutional Ethical Committee for Stem Cell Research via reference No. IC-SCR/RM/50/22.

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