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Research Article

Role of rTMS and Expression Levels of Serum Growth Factors Along with Neurophysiological Markers in Ischemic Stroke Recovery: A Double Blind, Parallel Group, Sham Controlled Randomized Study

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Abstract

Background: The therapeutic benefits and efficacy of repetitive trans cranial magnetic stimulation along with physical therapy in exerting the cellular and molecular aspects, and changes in neurophysiological markers during the functional recovery in ischemic stroke patients have not been fully elucidated. The aim of this study is to determine the role of serum growth factors levels as a surrogate marker, using 1Hz rTMS with conventional physiotherapy in patients with ischemic stroke.

Methods: Study participants with first ever ischemic stroke (N = 96), onset within 15 days were randomized after a run-in period of 75 ± 7 days along with standard physical therapy to receive 10 sessions of real 1Hz rTMS (N = 47) on contra lesion premotor cortex or to sham stimulation (N = 49) for 2 weeks. Participants, investigators and outcome assessors were blinded. The study outcomes were to measure changes in the expression levels of peripheral serum growth factors VEGF and BDNF and neurophysiological changes at third month.

Results: Modified intention to treat analysis showed significant up regulation in the mean levels of serum VEGF (797.24 ± 224.27) and BDNF (30.64 ± 9.64), P < 0.001 in Real rTMS Group. Trend of decrease in Resting Motor Threshold and an increase in Motor Evoked Potential in the affected hand was observed. Statistically significant negative correlation between motor evoked potential and mean VEGF (rho = -1.000, P < 0.001) in the affected hand in Real rTMS Group was seen.

Conclusion: Total ten sessions of 1Hz rTMS plus physical therapy on contralateral hemisphere resulted in up regulation of serum growth factors possible reflecting improved neuroplasticity.

Keywords: Ischemic Stroke; Transcranial Magnetic Stimulation; Serum Growth Factors; Motor Threshold; Motor Evoked Potential

Abbreviations

rTMS: Repetitive Trans Cranial Magnetic Stimulation; TMS: Trans cranial Magnetic Stimulation; BDNF: Brain Derived Neurotrophic Factor; VEGF: Vascular Endothelial Growth Factor; AIIMS: All India Institute of Medical Sciences; ICMR: Indian Council of Medical Research; RMT: Resting Motor Threshold; MEP: Motor Evoked Potential; APB: Abductor Pollicis Brevis muscle; mBI: modified Barthel Index; CT: Computerized Tomography; CONSORT: Consolidated Standards of Reporting Trials; t-PA: recombinant tissue Plasminogen Activator; IFCN: International Federation of Clinical Neurophysiology; MT: Motor Threshold; Unaff: Unaffected; Aff: Affected

Introduction

Stroke is the leading cause of adult neurological disability, affecting one in six people worldwide. Although various acute rehabilitative measures have been introduced to improve the outcome, but full recovery is still questionable. Although reperfusion treatment benefits 20% of ischemic stroke patients, their effectiveness does not exceed 50-60% [1]. Although various functional enhancing management techniques like physical therapy and conventional tools are available but till date no validated rehabilitative approach has been introduced which could restore the impaired functions by a complete recovery of the damaged tissue [2].

As new neuroprotective approaches are limited for clinicians and researchers, a need for new targets for the medium and long term prognosis of ischemic stroke is needed depending upon the size of infarction, speed and efficacy of the acute phase, therapeutic measures and the degree of neuro repair processes mediated by cerebral plasticity mechanism called cellular plasticity and its stimulation using growth factors causing neurogenesis, synaptogenesis mechanism and constitute a more translation therapeutic approach from the lab to the bedside [1]. Few studies have evaluated the motor recovery pattern using non-invasive approach, despite of that more research in understanding the cellular mechanism and restorative activities using rTMS is needed [3,4].

To overcome this problem, neuroplasticity plays a vital role in the functional prognosis of stroke patients, and the same could be modulated by non-invasive neuro rehabilitation tool called trans cranial magnetic stimulation. This interactive mechanism between TMS and neurons produces wide range of electrophysiological, biochemical and molecular, cellular changes and also affects behavior, mood, memory, myelination and neuroplasticity [5].

At molecular level, certain neurotrophins like BDNF, VEGF are found to be up regulated in the first 4 weeks after stroke, creating a "neuroplastic milieu" in which cortical remodeling within the intact brain is optimized [6].

Various animal studies have studied the post ischemic molecular events in which growth of new synapses and dendritic branching modifies the intact sensorimotor cortex and subcortical structures contributing to the recovery of cognitive and motor performance but understanding the neuroplasticity and neurogenesis model in patients with ischemic stroke recovery is yet to be studied [7-9]. Few researchers have studied the reduction in the oxidative stress and enhancement in the proliferation and signaling pathways using extremely low magnetic field with physical rehabilitation with few endothelial biomarkers in acute ischemic stroke patients [10] but understanding the neuro pathophysiology and neurogenesis during Ischemic Stroke recovery stage is still lacking.

Therefore, the aim of this study was to assess the levels of serum growth factors in studying the cortical excitability, neurogenesis measured through neurophysiological parameters by administrating low frequency rTMS as a non-invasive stimulation approach in combination with conventional physical therapy in sub-acute ischemic stroke patients.

Rationale

The rationale of the study was to conduct the clinical translation research using rTMS along with physiotherapy at sub-acute recovery stage, which is considered as the golden period for the initiation of exogenous restorative therapies. Endogenous repair reaches peak levels at this stage followed by the functional reorganization which enhances neural plasticity in the brain [11].

Materials and Methods

The study selection criteria, sample size estimation, methodology and rehabilitative protocol has been followed based on the our primary study objective [12] following CONSORT Guidelines (Figure 1), which was based on the functional outcome

of ischemic stroke patients only. The growth factor estimation and neurophysiological outcomes are the part of our secondary outcomes of our research in correlation to the functional recovery.

Participants, setting and study design

The study was conducted at the All-India Institute of Medical Sciences (AIIMS), New Delhi. This was a single center, randomized, parallel group, double blind, and sham-controlled trial. AIIMS Ethics committee approved the study protocol. The trial was registered at the Clinical Trial Registry India (CTRI/2016/02/006620) and funded by Indian Council of Medical Research (ICMR), India. All the participants or their caregivers gave written informed consent. We included patients aged 18-75 years, with first ever-acute ischemic stroke, within last 15 days documented by CT/MRI scan of the head.

Procedure

Pre-randomization run-in period

A trained licensed physiotherapist provided the standard physical therapy to all recruited participants. All participants received a standard care, which includes passive, active and active assisted exercises. Participants were encouraged to continue motor training at home after discharge and total number of hours of an individual exercise was monitored.

Growth factor estimation

Approximately 5ml non- fasting intravenous blood sample was withdrawn in an EDTA vial for the estimation of serum VEGF (pg/ml) and BDNF (ng/ml) at the time of recruitment, pre-rTMS and post rTMS period. Samples were then immediately centrifuged (1500g/15minutes, and serum was stored at -70°C until assayed. Both VEGF (pg/ml) and BDNF (ng/ml) levels were measured using standard quantitative sandwich ELISA (Quantikine, USA) kits obtained from R and D Systems. Samples from each individual were analyzed in a triplicate.

Interventions

Low frequency rTMS was performed using Magstim Rapid stimulator, Magstim Ltd, UK equipped with air-cooled figure of eight coil (70 mm) i.e., biphasic pulse. The resting motor evoked potential (MEP) was ascertained using an electromyogram, recording from the Abductor Pollicis Brevis (APB) in accordance to the International Federation of Clinical Neurophysiology (IFCN) recommendations [14]. The coil was placed tangentially to the scalp over the hand area of the primary motor cortex to calculate hot spot. Hot spot was defined as the location on the scalp where stimulation of a slightly supra threshold intensity elicited the largest motor evoked potential (MEP) in the APB muscle. After the hot spot was identified, resting motor threshold was determined using the lowest stimulus intensity to produce motor evoked potential of > 50μV peak-to-peak amplitude in 5 out of 10 subsequent trails. If no MEP was obtained at the time of hot spot calculation in the affected ipsilesion M1, then the hotspot was defined as the symmetric location to the contra lesion M1.The stimulation parameters were chosen in accordance with the safety guidelines for Rtms [14].

Total 750 pulses, 75 trains using low frequency (1Hz) with inter train interval of 45 seconds at calculated intensity of 110% resting motor threshold (RMT) (Fc3/Fc4), was administered to the randomized patient. Localization was done using 10-20 EEG methods. Sham rTMS pulses were administered using the same stimulation parameters over the contra-lesion premotor cortex area with the figure of eight coil angled at 90 degrees from the scalp. Patient was aroused throughout the rTMS administration.

The rTMS sessions on each day was followed by 45-minute conventional physical therapy regime given by a trained physiotherapist.

Monitoring for complications

All participants were monitored for any adverse events. A checklist of previously reported side effects was used to report any possible adverse events.

Study outcomes

The co-primary efficacy outcomes were changes in serum growth factors level VEGF (pg/ml), BDNF (ng/ml) measured at 3 months of ischemic stroke onset. The secondary efficacy outcomes were changes in the physiological markers Resting Motor Threshold, Motor Evoked Potential and change in the MEP duration during rest at 3 months stroke onset.

Statistical analysis

Statistical analysis was performed using STATA version 14.1 and was based on modified intention to treat principle. Normal distribution was tested using Kolmogorov - Smirnov statistic. Study data was not following normal distribution; hence non-parametric tests were applied. For Categorical data, 2 x 2 table was generated. Chi-square test/Fisher's exact test was applied to compare the properties in the two groups. Between groups comparisons were carried out using Wilcoxon's rank sum test. Wilcoxon signed rank

test was used to assess changes in the scores within the same group. Non-parametric independent test analysis was also done using log algorithm. Nemar test was applied for the categorical variable. For serum estimation, non- parametric independent t-test was applied. For correlation analysis, spearman correlation was applied.

Results

Between August 2012 and February 2016, 445 participants with acute ischemic stroke were screened and 139 participants fulfilling the eligibility criteria were recruited into the pre- rTMS run-in phase. During the run-in period, 35 participants withdrew consent and 4 died after discharge from the hospital. Randomization was done in 100 participants (Figure 1). Four participants were excluded after randomization (consent withdrawn after randomization). The baseline characteristics were comparable between Real rTMS (n = 47) and sham rTMS (n = 49). Mean age in Real rTMS arm was 54.85 ± 13.39 vs. 52.89 ± 14.95 years in Sham rTMS arm. There were more of male compared to female in both the groups (Table 1). Mean serum biomarkers VEGF (pg/ml) and BDNF (ng/ml) were comparable. All study participants received standard of care and at least one session of real or sham rTMS. Seven participants in real rTMS were lost to follow up. One participant had seizure and un-blinding was done for that participant and was managed symptomatically. Modified intention to treat analysis was done for 47 participants in real and 49 participants in sham rTMS arm.

Figure 1

Variable	Sham rTMS N = 49 Mean ± S.D 95% CI	Real rTMS N = 47 Mean ± S.D 95% CI	P
Age	52.89 ± 14.95 48.60 - 57.19	54.85 ± 13.39 50.91 - 58.78	0.50
Sex N (%) Male Female	 34(69) 15(31)	 33(77) 14(30)	0.93
Hypertension N (%)	32(65)	35(74)	0.60
Diabetes N (%)	13(26)	11(23)	0.45
Smoking N (%)	16(33)	15(32)	0.55
Tobacco Chewing N (%)	6(12)	4(8)	0.39
IV-tPA N (%)	6(12)	6(12)	0.59
Stroke Subtype N (%) Large artery Small artery Lacunar Cardio-embolic Others	 12(25) 4(8) 2(4) 7(15) 24(48)	 12(26) 3(6) 1(2) 6(13) 25(53)	0.98
Onset to enrollment N (%) ≤ 7 8-15 Mean ± S.D.	 41(82) 6(12) 4.68 ± 1.26	 36 (72) 11(22) 4.96 ± 1.56	0.33
Onset to TMS N (%) 60-75 76-83 Mean ± S.D.	 8(16) 39(78) 77.34 ± 10.21	 16(32) 31(62) 77.51 ± 5.38	0.91
VEGF (pg/ml) Mean ± S.D.	721.37 ± 217.2	728.41 ±198.75	0.09
BDNF (ng/ml) Mean ± S.D.	15.48 ± 8.99	18.73 ± 10.79	0.11

Table 1: Showing Baseline characteristics between Real rTMS group, N = 47 and Sham rTMS group, N = 49.

Growth factors estimation

Compared to healthy controls, down regulation expression level of mean serum BDNF was seen. On the other hand, mean VEGF was elevated on 5th day at the time of recruitment (Table 2 and 3 and Figure 4 and 5).

	VEGF (pg/ml)		
S.No	**Real rTMS n = 47 Mean ± S.D Median (IQR)**	**Sham rTMS, n = 49 Mean ± S.D Median (IQR)**	**P Value**
Baseline	728.41 ± 198.75 780 (180)	721.37 ± 217.2 746.63 (283.4)	0.9*
Pre- rTMS	637.23 ± 187.65 658.2 (207)	655.80 ± 472.67 579.6 (476)	0.1*
Post rTMS	797.24 ± 224.27 824 (101)	673.04 ± 193.28 745 (188)	< 0.001
Healthy	435 ± 275.12 374 (357)		
Delta Vegf = Base. – post rTMS	-68.83 ± 245.65 -34.40 (179.6)	48.32 ± 298.10 87 (242.37)	0.01 #

Table 2: Table showing mean VEGF (pg/ml) level at various time points.

*log

#Ranksum

	BDNF (ng/ml)		
S. No.	**Real rTMS n = 47 Mean ± S.D Median (IQR)**	**Sham rTMS, n = 49 Mean ± S.D Median (IQR)**	**P Value**
Baseline	18.73 ± 10.79 20.42 (16.77)	15.48 ± 8.99 15.3 (15.45)	0.11
Pre- rTMS	25.06 ± 12.67 22.52 (19.41)	22.73 ± 6.94 21.58 (10.62)	0.26
Post rTMS	30.64 ± 9.64 31.95 (10.50)	25.12 ± 10.11 24.64 (11.94)	< 0.001
Healthy	23.0 ± 11.0 20 (14) N = 50		
BDNF delta	-11.90 ± 11.35 -10.98 (14.34)	-9.64 ± 10.21 -8.21 (11.82)	0.33

Table 3: Table showing mean BDNF (ng/ml) level at various time points.

Non-parametric independent t-test showed significant increase in the mean VEGF (pg/ml) level in Real rTMS Group 797.24 ± 224.27 vs. 637.04 ± 193.28, p < 0.001 (Figure 2). Statistically significant change in the magnitude i.e., delta level of VEGF (pg/ml), -68.83 ± 245.65) was higher in the Real rTMS arm vs. 48.32 ± 298.10 in Sham rTMS arm.

Statistical significant increase in the mean level of serum BDNF (pg/ml) was seen in Real rTMS Group, 30.64 ± 9.64 vs. Sham r TMS, 25.12 ± 10.11, p < 0.001(Figure 3).

Figure 2: Whisker plot showing median distribution of serum VEGF (pg/ml) change from pre to post rTMS between Real rTMS group, N = 47 and sham rTMS group, N = 49.

Figure 3: Whisker plot showing median distribution of serum BDNF (ng/ml) change from pre to post rTMS between Real rTMS group, N = 47 and sham rTMS group, N = 49.

Figure 4: Whisker plot showing median distribution of serum VEGF (pg/ml) levels between Real rTMS, N=50 and sham rTMS, N = 50 in comparison to healthy controls at Baseline.

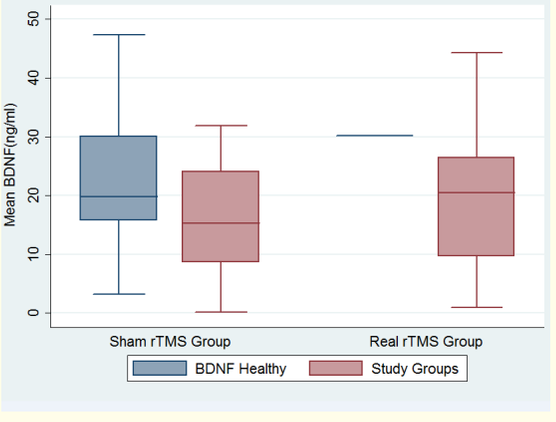

Figure 5: Whisker plot showing median distribution of serum BDNF (ng/ml) levels between Real rTMS, N=50 and sham rTMS, N = 50 in comparison to healthy controls at Baseline.

Neurophysiological markers

Resting motor threshold

Non-parametric independent t-test showed a trend of decrease in the RMT in the affected hand in the Real rTMS Group 69.40 ± 22.34 vs. 74.35 ± 14.72 in Sham rTMS Group, P = 0.31. Compared to the control arm, total N = 14 patients have shown change in the motor threshold level in active arm whereas a vice versa effect with an increase in the Resting Motor Threshold in the unaffected hand was seen in the Real TMS Group 76.17 ± 13.09 vs. 73.64 ± 16.00, P = 0.35 in Sham rTMS Group. Compared to the control arm, total N = 23 patients in Real rTMS showed change in the motor threshold level (Table 4 and 5).

	Resting Motor Threshold (RMT) Affected Hand		
S. No.	**Real rTMS Mean ± S.D Median (IQR)**	**Sham rTMS Mean ± S.D Median (IQR)**	**P Value**
Pre- rTMS	93.30 ± 9.33 N = 13	84.80 ± 15.69 N = 21	0.11 #
Post rTMS	69.40 ± 22.34 N = 27	74.35 ± 14.72 N = 31	0.31

Table 4: Table showing mean Resting Motor Threshold in affected hand from pre to post rTMS intervention.

#ranksum.

	Resting Motor Threshold (RMT) Non-Affected Hand		
S. No.	Real rTMS Mean ± S. D	Sham rTMS, n Mean ± S. D	P Value
Pre- rTMS	69.30 ± 10.58 N = 40	70.92 ± 11.79 N = 41	0.52
Post rTMS	76.17 ± 13.09 N = 17	73.64 ± 16.00 N = 25	0.59

Table 5: Table showing mean Resting Motor Threshold in non-affected hand from pre to post rTMS intervention.

Motor evoked potential

Nonparametric independent t-test analysis showed a decreased in the motor evoked potential amplitude in the non - affected hand in Real TMS Group 189.66 ± 77.64 Vs. Sham rTMS Group which was 158.17 ± 82.82, P = 0.22 whereas an increase in the MEP in the affected hand was seen in both the Groups immediate after rTMS intervention, P = 0.83 (Table 6).

Motor Evoked Potential	**Sham rTMS**	**Real rTMS**	**P- Value**
Affected Hand Pre rTMS Mean ± SD	104.89 ± 64.66 N = 10	93.43 ± 77.16 N = 6	0.75
Post rTMS Mean ± SD	120.98 ± 64.30 N = 23	116.64 ± 58.16 N = 16	0.83
Non affected Hand Pre rTMS Mean ± SD	154.62 ± 85.87 N = 33	193.93 ± 93.32 N = 33	0.08*
Post rTMS Mean ± SD	158.17 ± 82.82 N = 25	189.66 ± 77.64 N = 17	0.22

Table 6: Table showing mean Motor Evoked Potential in both affected hand from pre to post

rTMS intervention.

*log value.

Absolute change in the MEP duration

Significant increase in the mean absolute change in the MEP duration (in milli seconds) in the affected hand was seen in the Sham rTMS Group 7.44 ± 2.58 vs. 5.62 ± 1.64 in Real rTMS Group, P < 0.01 at post rTMS, whereas no significant change in the unaffected hand was observed in either Group (Table 7).

Absolute change MEP duration (delta MEP duration)	Sham rTMS	Real rTMS	P- Value
Affected hand			
Pre rTMS Mean ± SD	5.9 ± 2.37 N = 10	5.00 ± 1.26 N = 6	0.40
Post rTMS Mean ± SD	7.44 ± 2.58 N = 23	5.62 ± 1.64 N = 16	0.01
Non affected Hand			
Pre rTMS Mean ± SD	7.14 ± 2.60 N = 34	7.11 ± 2.81 N = 34	0.96
Post rTMS Mean ± SD	7.91 ± 3.34 N = 24	7.67 ± 2.12 N = 17	0.79

Table 7: Table showing mean absolute change in MEP duration in affected hand from pre to post rTMS intervention.

Growth factors vs. clinical outcome

Referring to our previous primary objective study [12], a trend of negative correlation was seen in the mean expression level of BDNF(ng/ml) with absolute change in the clinical outcome NIHSS (r = -0.264, p = 0.06) in Sham Group whereas no changes could be seen in the mean VEGF (pg/ml) level in any group (Table 12-15).

Correlation of growth factors vs. neurophysiological outcomes

VEGF vs. Neurophysiological parameters

Spearman analysis showed significant negative correlation (r = -1.000, P < 0.001) of VEGF (pg/ml) with motor evoked potential in the affected hand in Real rTMS Group, N = 05 compared to Sham rTMS Group (r = 0.2822, P = 0.37), N = 12 (Table 8 and 9).

			Sham rTMS Group - VEGF (pg/ml)					
Variable		**VEGF (pg/ml)**	**MT-post-unaff**	**MT-post-aff**	**MEP-post-unaff.**	**MEP-post-aff**	**Delta-MEP width-unaff.**	**Delta-ME duration-unaff.**
MT-post-unaff.	rho P	0.2544 0.4249	1.0000					
MT-post-aff.	rho P	-0.0426 0.8955	-0.0283 0.9305	1.0000				
MEP-post-unaff	rho P	-0.5221 0.0817	-0.1547 0.6313	-0.1129 0.7269	1.0000			
MEP-post-aff	rho P	0.2822 0.3742	0.1125 0.7278	-0.2822 0.3742	0.4246 0.1689	1.0000		
Delta-MEP duration-unaff.	rho P	-0.3298 0.2952	-0.6590 0.0198*	0.1454 0.6521	0.5961 0.0408	0.0847 0.7936	1.0000	
Delta-MEP duration-aff.	rho P	-0.1137 0.7250	-0.3752 0.2294	-0.2842 0.3707	0.5972 0.0403	0.1661 0.6059	0.6963 0.0119	1.0000

Table 8: Table showing correlation between serum VEGF and neurophysiological markers from pre to post in Sham rTMS Group. Total observation, N = 12.

			Real rTMS Group - VEGF (pg/ml)					
Variable		**VEGF (pg/ml)**	**MT-post-unaff.**	**MT-post-aff**	**MEP-post-unaff.**	**MEP-post-aff**	**Delta-MEP duration-unaff**	**Delta-MEP duration-unaff.**
MT-post-unaff.	rho P	-0.2000 0.7471	1.0000					
MT-post-aff.	rho P	0.2000 0.7471	0.8000 0.1041	1.0000				
MEP-post-unaff	rho P	-0.3000 0.6238	-0.3000 0.6238	-0.3000 0.6238	1.0000			
MEP-post-aff	rho P	-1.0000 0.0000	0.2000 0.7471	-0.2000 0.7471	0.3000 0.6238	1.0000		
Delta-MEP duration-unafff	rho P	0.6000 0.2848	-0.4000 0.5046	0.1000 0.8729	0.5000 0.3910	-0.6000 0.2848	1.0000	
Delta-MEP duration-aff	rho P	0.1026 0.8696	0.4104 0.4925	0.8208 0.0886	-0.0513 0.9347	-0.1026 0.8696	0.3591 0.5528	1.0000

Table 9. Table showing correlation between serum VEGF and neurophysiological markers from pre to post in Real rTMS Group rTMS intervention.

Total observation, N = 5.

BDNF vs. Neurophysiological parameters

Spearman correlation showed statistically positive correlation (r = 0.9000, P = 0.03) of BDNF (ng/ml) with delta MEP duration in the unaffected hand in Real rTMS Group vs. Sham Group (r = -0.2465, P = 0.43) (Table 10-15).

			Real rTMS Group - BDNF (ng/ml)					
Variable		**BDNF (pg/ml)**	**MT-post-unaff.**	**MT-post-aff**	**MEP-post-unaff.**	**MEP-post-aff**	**Delta-MEP duration-unaff.**	**Delta-MEP duration-unaff.**
MT-post-unaff.	rho P	-0.3000 0.6238	1.0000					
MT-post-aff.	rho P	0.0000 1.000	0.8000 0.1041	1.0000				
MEP-post-unaff	rho P	0.8000 0.1041	-0.3000 0.6238	-0.3000 0.6238	1.0000			
MEP-post-aff	rho P	-0.3000 0.6238	0.2000 0.7471	-0.2000 0.7471	0.3000 0.6238	1.0000		
Delta-MEP duration-unaff	rho P	0.9000 0.0374	0.4000 0.5046	0.1000 0.8729	0.5000 0.3910	-0.6000 0.2848	1.0000	
Delta-MEP duration-aff	rho P	0.2052 0.7406	0.4104 0.4925	0.8208 0.0886	-0.0513 0.9347	-0.1026 0.8696	0.3591 0.5528	1.0000

Table 10. Table showing correlation between serum BDNF and neurophysiological markers from pre to post in Real rTMS Group.

Total Observation, N = 05.

			Sham rTMS Group - BDNF (ng/ml)					
Variable		**BDNF (pg/ml)**	**MT-post-unaff.**	**MT-post-aff**	**MEP-post-unaff.**	**MEP-post-aff**	**Delta-MEPduration-unaff.**	**Delta-MEPduration-unaff.**
MT-post-unaff.	rho P	0.3789 0.2244	1.0000					
MT-post-aff.	rho P	0.0493 0.8791	-0.0283 0.9305	1.0000				
MEP-post-unaff	rho P	-0.2032 0.5266	-0.1547 0.6313	-0.1129 0.7269	1.0000			
MEP-post-aff	rho P	-0.0701 0.8287	0.1125 0.7278	-0.2822 0.3742	0.4246 0.1689	1.0000		
Delta-MEPduration-unaff.	rho P	-0.2465 0.4399	-0.6590 0.0198	0.1454 0.6521	0.5961 0.0408	0.0847 0.7936	1.0000	
Delta-MEPduration-aff.	rho P	0.0000 1.000	-0.3752 0.2294	-0.2842 0.3707	0.5972 0.0403	0.1661 0.6059	0.6963 0.0119	1.0000

Table 11: Table showing correlation between serum BDNF and neurophysiological markers from pre to post in Sham rTMS Group.

Total observation, N = 12.

			Real rTMS Group – BDNF (ng/ml)					
Variable		**BDNF (ng/ml)**	**Delta mBI**	**Delta mRS**	**Delta NIHSS**	**Delta FMA Upper Extremity**	**Delta FMA Lower Extremity**	.
Delta mBI	rho P	0.2011 0.1753	1.0000					
Delta mRS	rho P	-0.0008 0.9956	0.3043 0.0376	1.0000				
Delta NIHSS	rho P	0.0274 0.8551	-0.6028 0.000	-0.2809 0.0558	1.0000			
Delta FMA Upper Extremity	rho P	0.0088 0.9530	-0.0782 0.6014	-0.5998 0.0000	0.0807 0.5897	1.0000		
Delta FMA Lower Extremity	rho P	0.0547 0.7152	-0.0049 0.9739	-0.4364 0.0022	-0.0628 0.6749	0.6401 0.000	1.0000	

Table 12: Table showing correlation between serum BDNF (ng/ml) and absolute change(delta) of clinical outcomes from pre to post in Real rTMS Group, N = 47.

Total Observation, N = 47.

		Sham rTMS Group – BDNF (ng/ml)					
Variable		**BDNF (ng/ml)**	**Delta mBI**	**Delta mRS**	**Delta NIHSS**	**Delta FMA Upper Extremity**	**Delta FMA Lower Extremity**
Delta mBI	rho P	-0.0139 0.9245	1.000				
Delta mRS	rho P	0.0084 0.9542	0.1495 0.3054	1.0000			
Delta NIHSS	rho P	-.0.2640 0.0668	-0.4555 0.0010	-0.3307 0.0203	1.0000		
Delta FMA Upper Extremity	rho P	-0.1159 0.4278	0.0737 0.6148	-0.6460 0.000	0.0712 0.6271	1.0000	
Delta FMA Lower Extremity	rho P	-0.1998 0.1688	0.0480 0.7434	-0.5084 0.0002	0.2726 0.0581	0.4033 0.0041	1.000

Table 13: Table showing correlation between serum BDNF (ng/ml) and absolute change(delta) of clinical outcomes from pre to post in Sham rTMS Group.

Total Observation, N = 49.

		Real rTMS Group - VEGF (pg/ml)					
Variable		**VEGF (ng/ml)**	**Delta mBI**	**Delta mRS**	**Delta NIHSS**	**Delta FMA Upper Extremity**	**Delta FMA Lower Extremity**
Delta mBI	rho P	0.0391 0.7943	1.000				
Delta mRS	rho P	0.0163 0.9132	0.3043 0.0376	1.0000			
Delta NIHSS	rho P	-.0.0215 0.8859	-0.6028 0.000	-0.2809 0.0558	1.0000		
Delta FMA Upper Extremity	rho P	0.1412 0.3439	-0.0782 0.6014	-0.5998 0.0000	0.0807 0.5897	1.0000	
Delta FMA Lower Extremity	rho P	0.0874 0.5591	-0.049 0.9739	-0.4364 0.0022	-0.0628 0.6749	0.6401 0.0000	1.000

Table 14: Table showing correlation between serum VEGF (pg/ml) and absolute change(delta) of clinical outcomes from pre to post in Real rTMS Group.

Total Observation, N = 47.

		Sham rTMS Group - VEGF (pg/ml)					
Variable		**VEGF (ng/ml)**	**Delta mBI**	**Delta mRS**	**Delta NIHSS**	**Delta FMA Upper Extremity**	**Delta FMA Lower Extremity**
Delta mBI	rho P	0.1052 0.4720	1.000				
Delta mRS	rho P	0.1573 0.2803	0.1495 0.3054	1.0000			
Delta NIHSS	rho P	0.1962 0.1768	-0.4555 0.0010	-0.3307 0.0203	1.0000		
Delta FMA Upper Extremity	rho P	-0.0769 0.5994	0.0737 0.6148	-0.6460 0.0000	0.0712 0.6271	1.0000	
Delta FMA Lower Extremity	rho P	-0.0634 0.6652	0.0480 0.7434	-0.5084 0.0002	0.2726 0.0581	0.4033 0.0041	1.000

Table 15: Table showing correlation between serum VEGF(pg/ml) and absolute change(delta) of clinical outcomes from pre to post in Sham rTMS Group.

Total Observation, N = 49.

Adverse events

One participant in the real TMS arm developed seizure (generalized tonic clonic seizure) 18 hours after the fourth session and about 1 h after waking from sleep in the morning. CT scan of head did not reveal any fresh changes. He was treated with phenytoin and the event was reported to Institute's Ethics Committee. The participant did not receive any more TMS sessions. No other adverse events were reported in the participants.

Discussion

In this Randomized controlled study, we measured the levels of serum growth factors VEGF and BDNF post administrating 1Hz rTMS onto the contra lesion hemisphere along with conventional physiotherapy in patients with sub acute ischemic stroke. We assessed the neurophysiological parameters, and a correlation of growth factors levels with neurophysiological parameters at third month.

Although various studies using human models have been conducted in understanding the motor and clinical recovery but very limited studies in humans have been done in understanding the cellular mechanisms mediating rTMS neuroprotective and restorative activities. We speculated that post stroke noninvasive treatment approach induced neurogenesis might play a future potential target for therapeutic intervention.

Post stroke acute recovery is known to be correlated with endothelial function. With the release of polypeptide growth factors after acute ischemic stroke, they are likely to play a vital role in both cellular and molecular process underlying wound healing and functional recovery in Stroke patients. Various observational studies reveal that VEGF mRNA up regulates under hypoxic conditions and elevated levels at during 7th day and 3rd month are found to be independently associated with good functional outcome [1,15] In our study, we found significant changes in the mean serum biomarkers VEGF and BDNF in an active arm i.e., Real rTMS Group compared with placebo arm i.e. Sham rTMS Group with conventional physical therapy in Stroke patients at third month.

VEGF is the most prominent hypoxia inducible angiogenic factor and a key mediator of angiogenesis and plays an important process leading to reperfusion of ischemic brain tissue after acute stroke, promoting neurogenesis, proliferation and differentiation of endothelial and neuronal progenitor cells [16-18]. Increased VEGF might have long term beneficial effects as a result of continued angiogenesis over several months [15]. Various studies are conducted in understating the VEGF expression various stroke recovery stages. Study conducted by Sobrino., *et al*. [1]. had shown high serum VEGF level at day 7, and at 3 months which is directly associated with good functional outcome. Another study conducted

by Lee and his colleagues [19] showed positive correlation of serum VEGF with long term prognosis in patients with acute ischemia. Another study conducted by Baba and his team [20] administered electrical stimulation for seven days in adult Wistar rats. Significant up regulation of both VEGF and BDNF was seen. Ours is the first study in assessing the role of rTMS using 1Hz frequency with conventional physical therapy using large sample size of ischemic stroke patients. Compared to healthy controls, higher expression of mean VEGF level was seen at the time of recruitment, signifying good prognosis. Results in our study have shown significant up regulation of mean VEGF level in Real rTMS Group. Referring to our previous published study, rTMS intervention has shown significant improvement in the magnitude of functional outcome in the active TMS arm [12]. Although, no significant correlation of mean VEGF with functional outcome was seen but up regulation of mean VEGF at immediate rTMS could justify the role angiogenesis which might play an essential role in the pathophysiology of stroke recovery mediated by rTMS.

Another neurotropic factor called BDNF is known to be involved in ischemia induced neurogenesis processes, increases recruitment of endogenous progenitors to injured brain regions, mediating repair mechanisms and neuronal plasticity in various brain disorders. It also induces synaptogenesis, morphogenesis, plasticity of dendritic spines, resulting in synapses with functionality [21,22], and also plays an important role as an effective indicator for rehabilitation interventions [23]. Supportive evidence-based studies suggests that rTMS causes enhancement of peripheral BDNF levels in patients suffering from Depression. In healthy human subjects, it has shown activation of BDNF signaling pathways [24-26]. There is accumulating evidence on the role of BDNF in therapeutic effects using 1Hz rTMS. BDNF polymorphism (val66met) negatively influences the effect of rTMS on upper limb weakness. The changes in blood levels of BDNF may be due to TMS induced alteration of BDNF-TrkB signaling in the brain. Recent study conducted by Niimi., *et al*. 2016 [27] investigated the molecular effects of rTMS using low frequency on serum levels of BDNF and other growth factors in stroke patients with upper limb hemiparesis. Statistically significant increase in the serum BDNF level was seen after two weeks combination therapy. A community-based cohort study [28]. having N = 3440 participants was conducted in understanding the association of serum VEFG and BDNF levels and based on their analysis it was concluded that lower serum BDNF and higher VEGF concentrations were associated with increased risk of incident.

Similar results could be seen in our study. Compared to healthy controls, lower levels of serum BDNF (ng/ml) were expressed. Immediate post RTMS showed significant elevated levels of BDNF in Real rTMS Group. Similar findings were seen in an observational study conducted by Sobrino., *et al*. 2020 [1], which reveals that elevated levels of serum endothelial growth factors are associated with good functional outcome at 3 months' ischemic stroke onset.

Ours is the first randomized controlled study with larger sample size to evaluate the association of both serum VEGF and BDNF levels in correlation to non- invasive approach with routine physical therapy intervention in understating neuroplasticity in ischemic stroke patients.

Neurophysiological Markers

The therapeutic effects of non-invasive stimulation i.e., Trans cranial magnetic stimulation could only be evaluated by applying either low (inhibitory) on contra lesion or high (excitatory) on postlesion hemisphere in stroke patients. rTMS has been known to restore the unbalanced inter hemispheric inhibition, which results in increased inhibition in the ischemic hemisphere and subsequent worsening in motor function whereas the neurophysiological effect of rTMS on neuronal activity has not been well explored. TMS is known to be used as an excellent non-invasive tool to map the electrophysiological changes in the cortical hemispheres. In addition, that, they are used to dissect physiological mechanisms underlying motor deficits, spontaneous motor recovery, and the effect of therapeutic interventions in ischemic stroke like motor threshold, motor evoked potential, latency period etc.

Neurophysiological changes occur both in acute and sub-acute phase of stroke mostly attributable to pathophysiological changes like reversal of diaschisis, resolution of edema etc. [29]. Neurophysiological parameters like MEP, amplitude, MEP duration could provide an important insight into the motor cortical function [30].

As MEP plays a main dependent variable in understanding the cortical function, recently the role of MEP duration was recently studied in both resting and facilitated state using various stimulus

intensities to understand the underlying physiological changes in MEP duration reflecting cortical and non-propriospinal mechanism involvement in stroke recovery [30].

In our study, MEP duration was assessed during resting state at a calculated intensity of 110% RMT and was correlated with the change in serum growth factors level to determine the cortical mechanisms and ceiling effects. MEP duration was measured from MEP onset latency to the time at which the activity returned to baseline. We determined the absolute change in the MEP duration during rest. Compared to control arm, statistically significant positive correlation of serum BDNF with delta MEP duration in the unaffected hand was seen in the active arm. Ours is the first study to correlate serum growth factor with change in the MEP duration in sub acute ischemic stroke patients using TMS as a main dependent factor in understanding the ceiling effect. Marisa and her colleagues measured an increase in duration of MEP during contraction in a group of healthy volunteers and on a group of patients with various neurological disorders. Compared to healthy controls, significant difference in the MEP duration at rest was seen [31].

An observational study was conducted to understand the physiological process underlying changes in MEP duration using TMS on healthy controls. Significant correlation of cortical inhibition and MEP duration was seen which might contribute as a future biomarker of motor cortical function [30].

Recent meta-analysis conducted by Zhang., *et al*. 2017 [32] reported that low frequency rTMS as an add on therapy improves functional recovery in Stroke patients. Trend of decrease in the cortico excitability, reflected in the form of Resting Motor Threshold and increase in the MEP in the affected hemisphere and vice versa has been found to be associated with motor recovery [33]. The pooled effects could also be seen in other supportive evidences [34-36] Our results are consistent with the study done by Du and his team [37], in which significant behavior neurophysiological correlation between cortico spinal excitability and motor improvement was seen at 3 months that indicates that enhanced motor cortical excitability in the stroke hemisphere is an important precondition for neural plasticity, which may allow the surviving neurons to reorganize in response to therapy. A trend of cortico excitability was seen in the affected arm in the neurophysiological parameters with a decrease in Resting Motor Threshold, increase in the Motor Evoked Potential in Real rTMS Group.

Limitations

Follow up randomized controlled studies with large sample size are required to understand the role of TMS intensity and its exhibited ceiling effect in ischemic stroke recovery.

Conclusions

The primary and secondary outcomes of the study have supported our study hypothesis. Higher levels of serum endothelial growth factors and significant change in the neurophysiological markers and its significant correlation with growth factors might play a vital role in understanding the neuroplasticity mechanism in stroke recovery.

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