Constraint Induced Movement Therapy as a Rehabilitative Strategy for Ischemic Stroke—Linking Neural Plasticity with Restoration of Skilled Movements

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Background: Stroke leads to devastating impact on health as well as quality of life making it one of the leading causes of disability. Restoring the functions of upper extremities after ischemic (ISC) stroke is one of the challenges for rehabilitation. Lack of trained professionals and accessibility to rehabilitation centers are limited in many counties. Constraint induced movement therapy (CIMT) has been practiced in regaining the functional activity following stroke. CIMT can be practiced with minimum clinical set up which makes it cost effective. However, the neural plasticity mechanism underlying the recovery with CIMT is not well understood. *Methods:* In the current study, we sought to investigate the extent to which CIMT helps in ameliorating neurological deficits in rat model of ISC stroke, induced by Endothelin-1 (ET-1). As well as to understand the cortical plasticity with Golgi-Cox staining and interhemispheric interaction with biotinylated dextran amine (BDA) following CIMT. Neurological deficits were identified within 24 hours of ET-1 infusion. *Results:* CIMT restored the impaired skilled movements after ISC stroke and improved the quality of fine movements. Golgi-Cox staining showed significant decrease in dendritic arborization in the injured motor cortex following ISC stroke. CIMT was efficient in reversing this effect as indicated by increased dendritic arborization especially in layer III pyramidal neurons. Also, the stroke induced asymmetry in dendritic length across both hemispheres was found to be reduced with CIMT. BDA tracing showed a re-establishment of the axonal connections between the hemispheres after CIMT. *Conclusions:* Implications of CIMT can be one of the promising and low cost rehabilitative technique for the individuals with upper limb movement deficits.

Key Words: Ischemic stroke—constraint induced movement therapy—reach to grasp task—neural plasticity—endothelin 1—functional recovery—motor cortex—layer III pyramidal neurons

Introduction

Stroke is the second leading cause of death and the third leading cause of disability.[1-3] Majority of stroke (70%-75%) is ischemic (ISC).[4-6] Approximately 12% of all strokes occurring in the population is below 40 years of age.[7] The financial repercussion associated with stroke and subsequent disability is substantial, hence the long-term disability has taken a toll on the socioeconomic status of the nation. Around 20% of survivors require hospital care after 3 months and 15%-30% of stroke survivors are permanently disabled.[4] However, in countries like India, the trained resources for rehabilitation in rural area are limited.[4,8] Especially when the stroke affects the earning member of the family, it leads to devastating

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consequences. The importance of rehabilitation has increased over the years to address these challenging facts.

Constraint induced movement therapy (CIMT) is one of the rehabilitation strategies that had proven the efficacy in stroke patients with motor disabilities[9,10] even in chronic stroke cases.[11,12] CIMT can also be used to improve the functional outcomes in children with hemiplegic cerebral palsy.[13] CIMT is designed to counter the phenomenon of "learned nonuse". Stroke patient tends to depend more on their unaffected limb for daily activities.[14-16] In CIMT, the patient's unaffected limb is restrained for a span of time (usually 6 hours daily) to perform motor tasks. The therapy can be given in home condition which makes the CIMT a cost-effective rehabilitation strategy. Understanding the underlying mechanism leading to the recovery following CIMT in stroke, might help in designing new therapeutic strategies and also in fine tuning the therapy in terms of timing and duration of treatment. It may also help in increasing the practice of advising CIMT to stroke as well as similar conditions to improve skilled movements.

The central nervous system has immense capacity to recover and adapt following any injury. This spontaneous recovery occurs in weeks to months following ischemia. It is known that the stroke leads to structural and functional reorganization of the brain, both the injured[17-19] and uninjured hemispheres.[20-23] The monkey experiments by Glees and Cole[24] showed focal injury to thumb representation, thus leading to remapping of adjacent cortex to take up the function of injured cortex. In humans, the transcranial magnetic stimulation studies have shown to be beneficial to the affected muscle following stroke.[19,25] Animal studies have also shown cortical remapping following stroke on similar lines.[26-28] These changes were not only constrained to injured cortex but also to uninjured cortex. An increase in the motor map area was found in the uninjured cortex following stroke.[15,29,30] Following stroke, structural modulation occurs in the peri-lesioned area.[18,31] In rodent studies,[18,32] increase in dendritic plasticity was found in the peri-infarct area. Increase in the spine formation indicating the increase in synaptic plasticity, occurs in 1-2 weeks postischemia.[33]

The learned nonuse reduces the innate neuroplasticity mechanisms in the injured hemisphere. This assumption is strengthened by an increase in the uninjured cortical map[15,29,30] which may increase the interhemispheric inhibition[34-36] and results in poor recovery.[37,38,39] Increased interhemispheric asymmetry leads to increase in interhemispheric inhibition resulting in motor deficits.[39] During CIMT, the unaffected limb is restrained so as to increase the use of affected limb, thus helping the patients to overcome the "learned nonuse".[9,16] Human studies have shown an increase in the contralateral (uninjured hemisphere) cortical motor map[40-42] after CIMT. However, underlying mechanisms may be better understood using animal models, where studies have demonstrated a functional improvement in motor performance,[43,44] enhanced neurogenesis,[44] and structural plasticity[45] with CIMT. The efficacy of CIMT was found to be dependent more on corticospinal projections from injured cortex (ipsilesional).[46,47]

As there are limited studies in the literature to understand the behavioral and morphological correlates of functional recovery with CIMT, the present study was designed using an Endothelin-1 (ET-1) ISC stroke rat model.[48] The application of ET-1 to near vicinity of middle cerebral artery (MCA) leads to occlusion of the MCA. This established model mimics the human condition with its small infarct, reperfusion and causes the specific behavioral abnormality.[64] Five days after the induction of ISC stroke, CIMT was applied continuously for 7 days with continued forced training during these 7 days period. Here, we investigated the effect of CIMT with forelimb immobilization[43,49] on the functional recovery of fine movements—reach to grasp task[50-52] and the morphological changes in layer III and layer V pyramidal neurons in injured and uninjured motor cortex. Interhemispheric connections were also studied with the help of biotinylated dextran amine (BDA).

Methods

Animals

Sprague Dawley male rats aged 3-4 months were bred and raised in Central Animal Research Facility of the institute. Experimental protocols were approved by the Institutional Animal Ethics Committee and care was taken to minimize discomfort to animals during all the procedures. The rats received food and water ad libitum. A total of 208 rats were used for the study out of which 16 rats died during the study and 19 rats were excluded for not performing the reach to grasp task. All rats in the study were trained for reach to grasp task. After the training they were randomly grouped into 6 groups, normal control (NC), vehicle Control (VC), ischemic (ISC), NC with the cast applied (NC + CIMT), VC with the cast (VC + CIMT), and ISC group with the cast (ISC + CIMT). NC groups (NC and NC + CIMT) did not receive any intervention throughout the study. The VC groups (VC and VC + CIMT) had received .9% saline intracortically and the ISC groups (ISC and ISC + CIMT) received theET-1 intracortically.

Surgery and Forelimb Immobilization

Rats were anesthetized with intraperitoneal injections of Ketamine (90-100 mg/kg body weight) and xylazine (5-10 mg/kg body weight) and mounted on a stereotaxic device (Stoelting Co. USA). Two microliter of ET-1 (#E7764-.1MG, 2000 pmol, .5 μL/min, Sigma-Aldrich, USA) was microinjected at stereotaxic co-ordinates AP: +1.6 mm, ML: ±5 mm, DV: −7.9 mm relative to bregma.[53]

Forelimb immobilization was performed on day 5. Cast was applied on the unaffected limb which is ipsilateral to the lesion, thus forcing the animals to rely on the affected limb (contralateral to the lesion) for postural support and movement. The plaster of paris cast was applied around the ribcage and unaffected forelimb.[43,49] A thick layer of cotton was placed before applying the cast to reduce the heat and damage to the fur. Forelimb immobilization was done for 7 days starting from day 5 after the ET-1 infusion. Minimum 3 rats were caged together to aid the grooming and the cast was reapplied if it displaced. The rats were excluded from the study if the cast was displaced more than 2 times. The study design is shown in Figure 1. The rats were trained for reach to grasp task after prior handling and habituation. Once the rats learned to perform the task successfully, they were grouped into 6 groups. The performance for reach to grasp task and cylinder test were video recorded using a Cineplex camera (30 fps, Plexon Inc, USA). Surgery was performed and the infusion of ET-1 was administrated intracortically for ISC and ISC + CIMT group. Twenty four hourrs after the surgery (D1), the behavior tests (cylinder test and Garcia scoring) were done. Recovery period of 5 days were given and the rats were not food deprived during the time. On the fifth day (D5), the reach to grasp task was done after a brief habituation. The task continued for 7 days. Meanwhile, the CIMT groups (NC + CIMT, VC + CIMT & ISC + CIMT), received the cast on day 5. On day 12 (D12), the cast was removed. All the groups were allowed to recover in home cage for 2 days. There was no reach to grasp training on these resting periods. Later, on day 15-day 17, rat's performance in reach to grasp task was tested for all the groups. Only, the performance on day 17 (D17) was taken for further analysis. Rats were sacrificed for morphological study on day 18.

Validation of ISC Stroke Model

Confirmation of ISC infarct was performed using 2,3,5-Triphenyltetrazolium chloride (TTC) staining after 24 hours. The 2 mm coronal sections were incubated with 1% buffered TTC[54] (PBS, pH 7.4; Sigma-Aldrich) for 30 minutes in the dark at room temperature.

Rats were subjected to gait assessment on day 1(D1) following stroke to analyze stride length of hind-limb and forelimb in NC and ISC groups. The average distance of 4 cycles was calculated for each limb separately. Severity of stroke was assessed using the Garcia score for neurological evaluation,[55] 24 hourrs after surgery (D1). All the 3 groups (NC, VC, and ISC) were tested for a battery of behavioral tests to assess the neurological deficit. Cylinder test for testing the asymmetry in spontaneous paw preference[56] was performed by allowing the rats to explore in a transparent Plexiglas cylinder for 3 minutes. The ipsilateral bias is calculated by the following formula

$$\text{Ipsilateral bias} = \frac{I - C}{I + C + B}$$

I = no. of ipsilateral (unimpaired) paw contacts with the wall; C = no. of contralateral (impaired) paw contacts with the wall; B = no. of both paw contacts with the wall simultaneously or nearly simultaneously.[57]

Reach to Grasp Task to Evaluate the Fine Motor Performance

During the reach training, 25-30 food pellets (rice crispies of 3 mm diameter and weighed approximately 25 mg, Bakery Machinery and Co., Bangalore) were given in the well, contralateral to the preferred paw. The rats, which learned to perform the task with 3 consecutive successful reaches, were included in the study. The performance of reach to grasp task was assessed for percentage of success, first attempt scoring and detailed analysis of movement components.[50] The percentage of success was scored using the formula given below, for first 20 pellets. The number of attempts to withdraw the pellet was noted for each pellet (number of reaches) despite of whether it was a successful event or not.

$$\%\ \text{of Success} = \frac{\text{number of successful reaches}}{\text{number of reaches}} \times 100$$

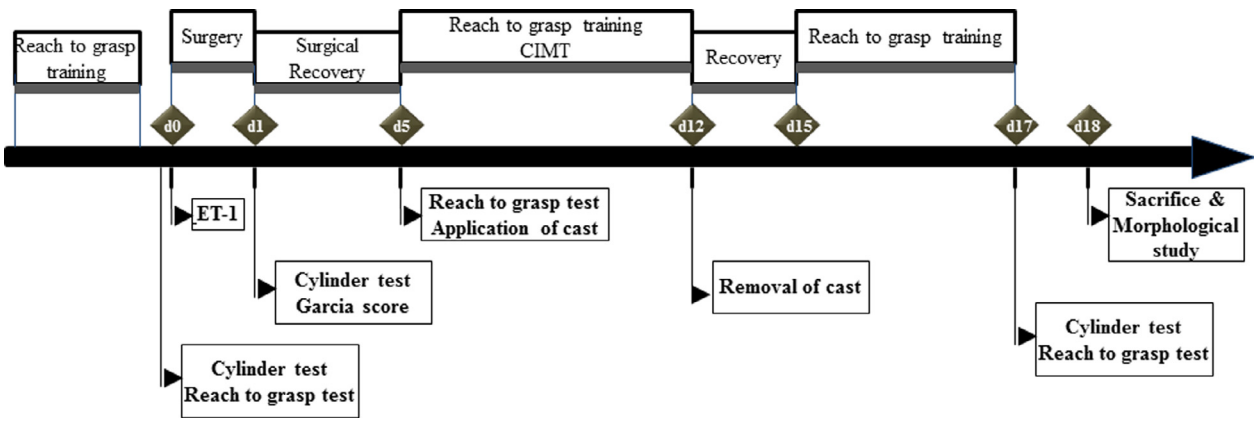

Figure 1. *Represents the timeline of the study design.*

If a pellet was successfully withdrawn in a single attempt, it was scored separately as first attempt success. The movement components scoring was described previously in an earlier study from the lab.[51,52] Eight movement components, including orient, limb lift, digit close, pronation, digit open, grasp, supination, and release were scored for 6 pellets. The number of abnormal movements made for each pellet was noted separately.

Golgi-Cox Staining to Study the Dendritic Arborization

After the behavioral evaluation, the rats were sacrificed, the brains were removed, washed with distilled water, and placed in freshly prepared Golgi-Cox solution[58] in amber bottles at 37°C for 24 hours. Brains were then kept in dark conditions at room temperature for 8-10ten days. One hundred and seventy micrometer coronal sections were taken onto gelatine coated slides and coded. The sections were treated with sodium carbonate (10%) followed by dehydration using increasing gradients of ethanol (70%-100%). The sections were flooded with Cedar oil (#96090, Sigma-Aldrich, USA) overnight, and then cleared with Xylene and mounted with DPX.

The reconstruction of pyramidal neurons from the rostral forelimb area (AP + 3.00 mm-AP + 2.04 mm)[53] was accomplished using NeuroLucida (Micro-brightfield Inc., Colchester, USA) under 40X objective (Olympus BX61 Microscope, Japan). The neurons from AgL (M1) region[59] on layer V and III were traced separately. Approximately, 10 neurons from layer III and 6 neurons from layer V of AgL (M1) region were reconstructed. Using NeuroLucida explorer (Micro-brightfield Inc., Colchester, USA), the Sholl analysis was carried out to calculate the dendritic length, number of branch points (nodes) and number of intersections made by the dendrite on the concentric circles drawn by considering the center of soma as reference point.[60] Both the hemispheres were considered separately for the study. Injured hemisphere (affected hemisphere) was the hemisphere contralateral to the impaired paw. The uninjured hemisphere (unaffected hemisphere) was ipsilateral to the impaired paw.

Anterograde Tracing of Axons

We used the high molecular weight biotinylated dextran amine (BDA, 10k Da, Molecular Probes Inc, USA) as anterograde tracer. Ten per cent buffered BDA solution (pH 7.4) was microinjected (1 μL, + 2.2 mm, ML: ±2.5 mm, DV: −2 mm) at the hemisphere which received the ET-1 infusion (i.e., injured hemisphere). After 30 days the rats were euthanized and transcardially perfused with saline followed by 4% buffered paraformaldehyde (pH 7.4). The brains were shelled out and post fixed for 48 hours in the same solution, followed by treatment with increasing gradients of sucrose (10%-30%). For further processing 40μm cryosections were collected onto gelatin coated slides. The labeling was performed using avidin-biotin immunoperoxidase method, where BDA traced fibers were visualized with the help of 3′-3′-diaminobenzidine.[61]

Statistical Analysis

Values were reported as mean±SEM and appropriate statistical measure was used for behavior experiments. The statistical significance of the effect of CIMT to reach to grasp task, was analyzed with 2-way ANOVA followed by Bonferroni multiple comparison post-tests. Dendritic morphology was analyzed by stringent nonparametric tests. The statistical significance of the effect of CIMT on dendritic length, intersections and nodes, in ischemia were calculated by Kruskal-Wallis nonparametric test followed by Dunn's multiple comparison test. Mann-Whitney *U* test (2-tailed) was used to analyze the statistical significance of CIMT in dendritic morphology across hemispheres.

Results

Validation of ISC Stroke Model in Sprague Dawley Rat

ET-1 infusion leads to ISC stroke as indicated by the infarct volume with TTC staining in the ISC brain Figure 2A, B compared to NC. Figure 2C represents the percentage of infarct volume (n = 5). ISC brain had increased neuronal death as indicated by an increased infarct volume (39.10% ± 6.607%). The motor cortex was found to be in ISC penumbra. The stride length (Fig 2D) of both forelimbs and hind limbs were observed to be significantly less in the ISC group when compared to the NC (Unpaired *t* test, $t_{(6)} = 10.6166$, $P < .0001$) indicating the gait abnormalities due to ISC stroke. ET-1 model of ISC stroke has shown asymmetry in spontaneous paw preference exhibiting significant ipsilateral bias while exploring the walls of Plexiglass cylinder in Cylinder test (Fig 2E) (Paired *t*-test, $t_{(6)} = 3.379$, $P = .0149$) showing the tendency of the animals to use the unimpaired limb more frequently to explore the wall after ISC injury. Garcia score (Fig 2F) indicated the severe neurological deficits on day 1 with minimal response to mild touch on the affected side of the body and to the vibrissae. Forelimb stretching and placing was also affected in the ISC group along with the spontaneous movement in the home cage 1-way ANOVA followed by Newmann-Keuls Multiple comparison post-test ($F_{(2,40)} = 26.89$) showed significant reduction in Garcia score in ISC (n = 15) rats when compared to NC (n = 14, $P < .0001$) and VC (n = 14, $P < .0001$).

Evaluation of Behavioral Outcome Following CIMT

Improvement in Percentage of Successful Retrieval of Pellets After CIMT

Reach success is a well-established measure of fine movements. Significant reduction in percentage of reach success (Fig 3A) was observed in ISC group in comparison

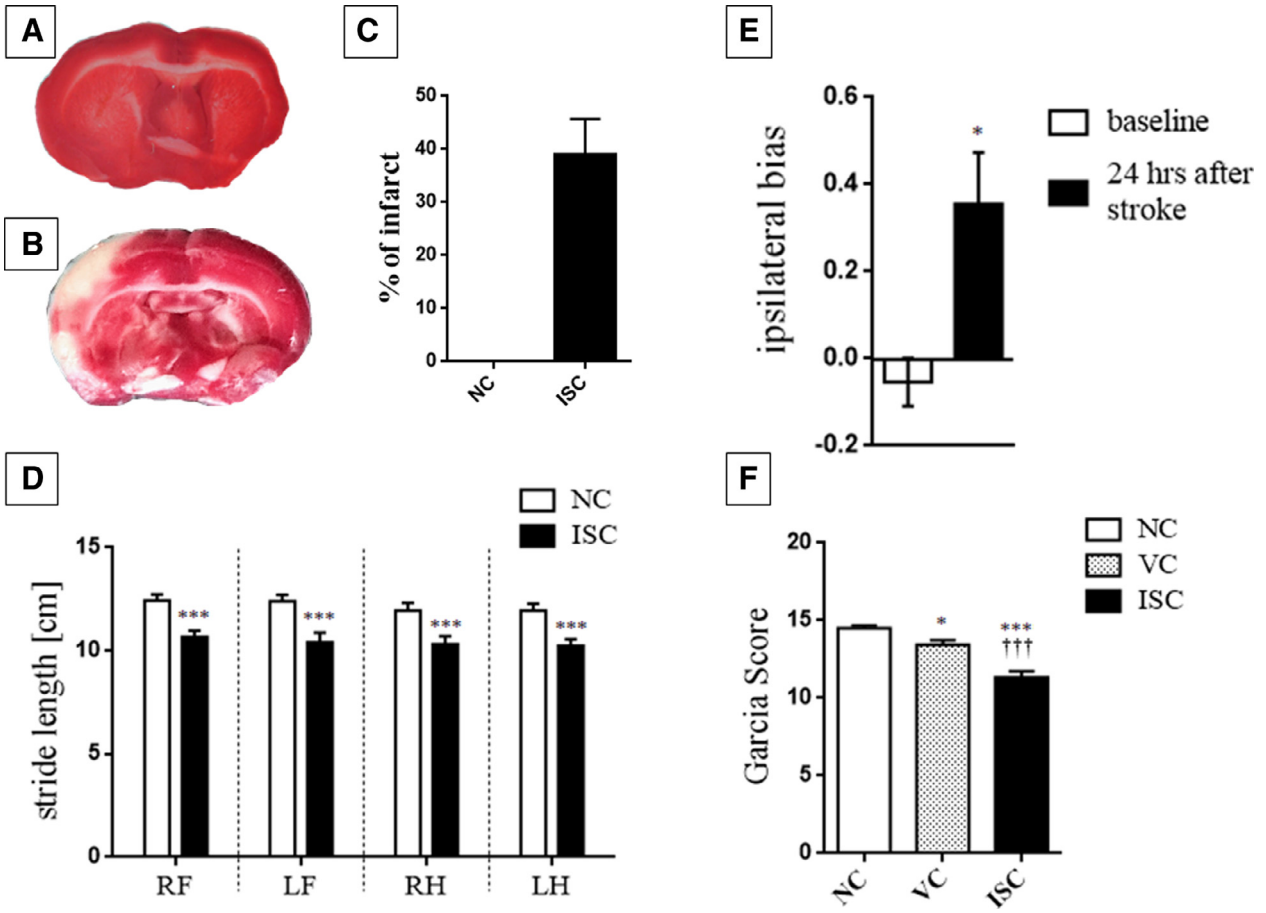

Figure 2. *Representative image showing infarct observed with 2,3,5-Triphenyltetrazolium chloride (TTC) staining in (A) normal control (NC) and (B) ischemic rats (ISC) after 24 hours of ET-1 induced injury. (C) Percentage of infarct volume. Behavioral validation of stroke using (D) Gait analysis for stride length. Data represents± SEM from NC (n = 9) and ISC (n = 10). (***P < .0001). (E) Cylinder test for spontaneous paw preference (*P = .0149). (F) Assessment of neurological deficit with Garcia scale. The ISC group (n = 15) showed significant decrease in score in comparison to NC (n = 14, ***P < .0001) and to VC (n = 14) (†††P < .0001). VC has also showed a significant decrease in comparison to NC (*P < .05).*

to NC ($P < .0001$), VC ($P < .0001$), NC + CIMT ($P < .0001$), and VC + CIMT ($P < .01$) on day 5 (2-way ANOVA followed by Bonferroni Multiple comparison post-tests $F_{(5,76)} = 16.55$). ISC + CIMT also showed significant difference in percentage of reach success to NC ($P < .0001$), VC ($P < .05$), and NC + CIMT ($P < .0001$) on day 5.There was no significant improvement in the ISC group performance on day 17 compared to NC ($P < .0001$), VC ($P < .0001$), NC + CIMT ($P < .0001$), VC + CIMT ($P < .0001$), whereas the stroke rats subjected to CIMT (ISC + CIMT), showed a significant increase in the percentage of reach success ($P < .0001$) indicating the improvement in fine movements. First attempt success also showed a similar trend indicating the improvement of reach accuracy with CIMT after ISC stroke. (Supplementary Fig 1)

Quality of Fine Movement in Reach to Grasp Task had Improved With CIMT in ISC Stroke Model

Two-way ANOVA followed by Bonferroni post-tests showed significant difference in the 8 movement components of the reach to grasp task across the days (Fig 3B) and across the groups (supplementary Fig 2) with CIMT, compared to ISC group. The movement score was significantly hampered in ISC group on day 5 in comparison to the baseline for digit close ($F_{(2,272)} = 65.99$, $P < .05$), pronation ($P < .001$), digit open ($P < .0001$), grasp ($P < .001$), supination ($P < .001$), and release ($P < .01$) indicating the deficit in skilled movement. Although we observed a slight improvement in the overall performance on day 17, there was significant impairment in digit open ($P < .05$), grasp ($P < .05$), supination ($P < .01$), and release ($P < .05$) compared to the baseline indicating chronic deficit. However the ISC + CIMT group showed significant improvement in the movement scores ($F_{(2,160)} = 37.48$), such as digit open ($P < .001$), grasp ($P < .001$), supination ($P < .001$), and release ($P < .01$) on day 5 when compared to baseline performance. We observed a temporal enhancement in the movement score, specifically grasp ($P < .05$) and supination ($P < .05$) on day 17 compared to day 5. No significant difference was observed for orient and limb lift in ISC group across the days indicating that

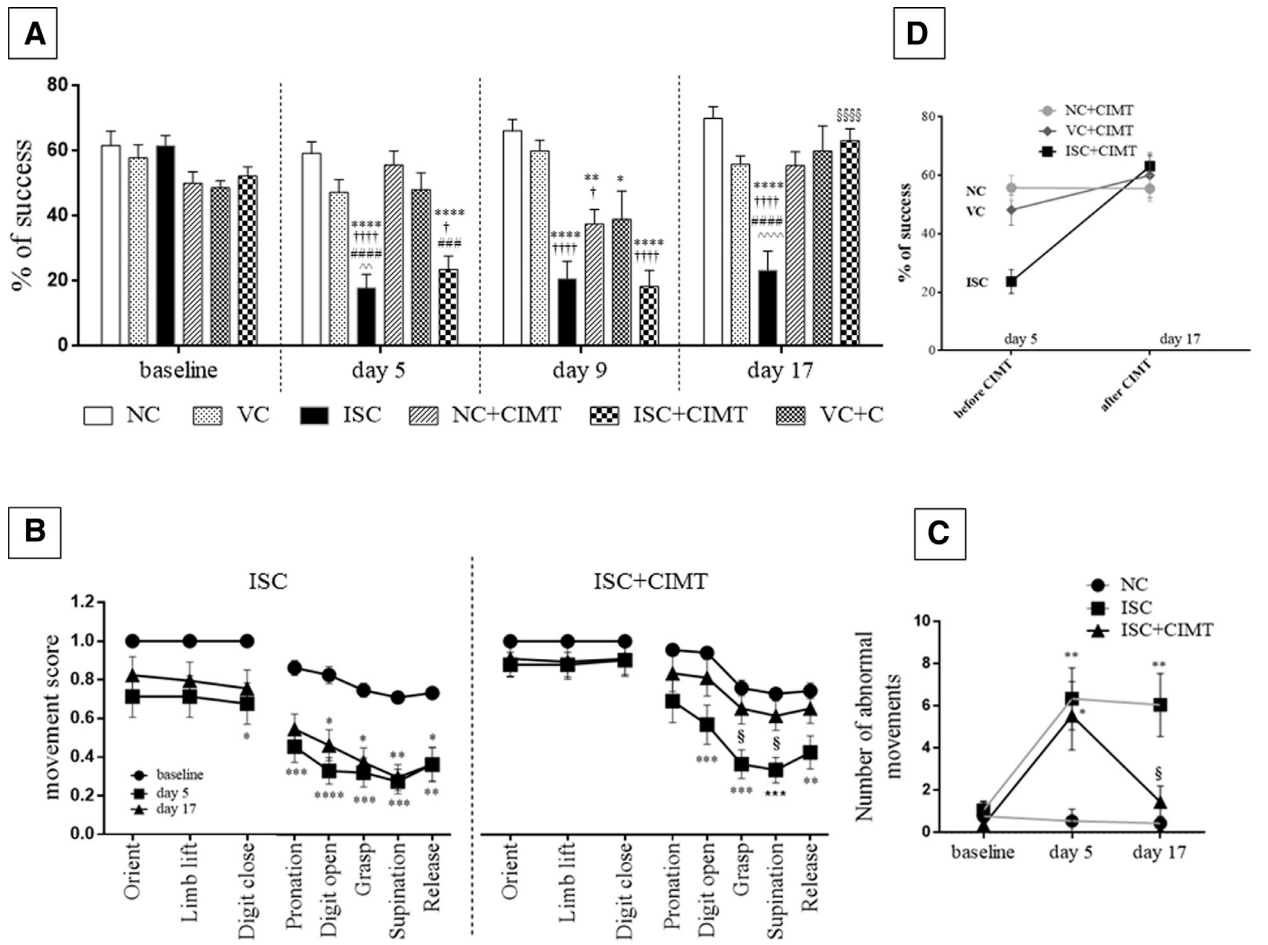

Figure 3. *(A) Success rate in reach to grasp task. Application of CIMT resulted in significant improvement in the performance on day 17 compared to ISC group (ssss*P < .0001). *(B) Improvement in the quality of fine movement after CIMT in ISC model. (C) Represents number of abnormal movements made during reach to grasp task at baseline, 5 days and 17 days after ISC stroke. Data represents mean±SEM (*P < .05). *(D) Summary of reach to grasp task showing improvement in fine movement over the period.*

the rats were able to orient and lift their limb, but failed to achieve the goal.

CIMT Played an Important Role in Ameliorating ISC Stroke Induced Abnormal Movement in Reach to Grasp Task

It was found that the abnormal/altered movements in the movement components (Fig 3 C) were high during day 5 in both ISC and ISC + CIMT group in comparison to NC group. But by day 17 the abnormal movements were reduced in ISC + CIMT group. Significant difference in score was observed in ISC group ($P < .01$) and ISC + CIMT group ($P < .05$) when compared to NC on day 5 (2-way ANOVA followed by Bonferroni post-tests, $F_{(2,34)} = 6.156$). The performance did not improve in ISC group and had significant differences when compared to NC ($P < .01$) and to ISC + CIMT ($P < .05$) on day 17 as shown in Figure 3D.

We observed an asymmetry in spontaneous paw preference for exploring the walls of cylinder as shown by an increase in ipsilateral bias in the ISC group in comparison to NC group on day 1. The deficit persisted throughout the study till day 17 in ISC group. However, in ISC + CIMT group the ipsilateral bias had reduced on day 17 as compared to the ISC (Supplementary Fig 3), but the difference was not significant. There was a significant difference in spontaneous paw preference in ISC group on day 1 and day 17 compared to NC group (2-way ANOVA followed by Bonferroni Multiple comparison post-tests, $F_{(5,60)} = 4.656$, $^{*}P < .05$; $^{***}P < .001$ in comparison with NC group). Also, significant difference was observed in ISC group when compared to NC + CIMT group on day 17 ($\#P < .05$).

Enhanced Dendritic Arborization With CIMT After ISC Injury

Increased Dendritic Arborization in Pyramidal Neurons of Layer III Motor Cortex After ISC Injury With CIMT

Ischemia lead to a decrease in total dendritic length in the injured cortex layer III pyramidal neurons (Fig 4A) as

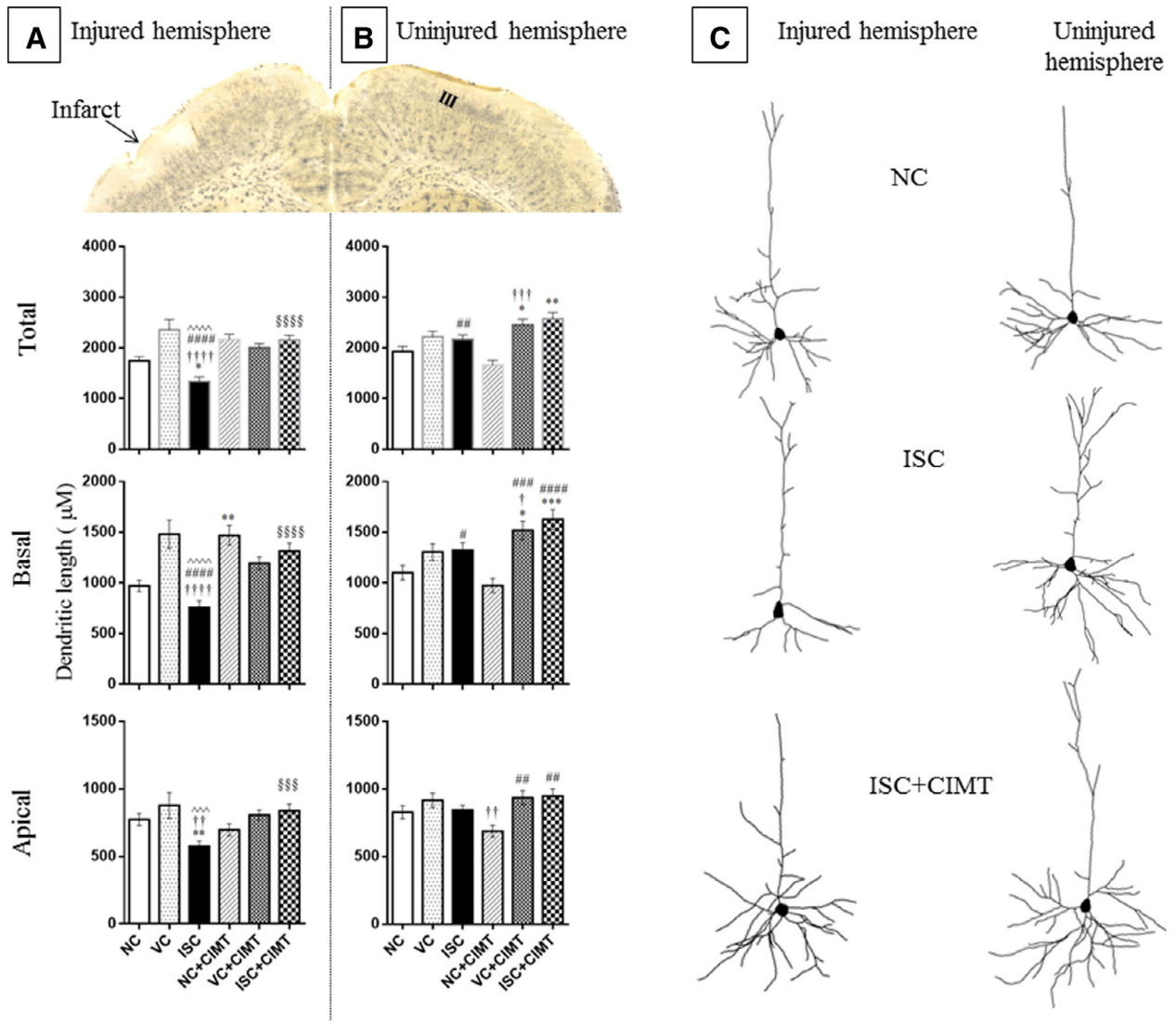

Figure 4. *Effect of CIMT on dendritic length of layer III pyramidal neurons of (A) injured and (B) uninjured hemisphere. Significant changes were observed in both the hemispheres. (C) Neuronal reconstruction of representative Golgi-impregnated pyramidal neurons from injured and uninjured motor cortex layer III with NeuroLucida. Significant difference in the total dendritic length of the ISC group in comparison to NC (*P < .05; **P < .01; *** P < .001).*

compared to NC ($P < .05$), VC ($P < .0001$), NC + CIMT ($P < .0001$), and VC + CIMT ($P < .0001$). The ISC group showed significant difference in basal dendritic length as compared to VC ($P < .0001$), NC + CIMT ($P < .0001$), and VC + CIMT ($P < .0001$). The difference was mainly at radial distance 30 μm-80 μm from soma (supplementary Fig 5). ISC group showed a significant reduction (notably at 80 μm-120 μm) in apical dendritic length as compared to VC ($P < .01$), NC + CIMT ($P < .01$), and VC + CIMT ($P < .001$). ISC + CIMT group had increased dendritic length in the injured hemisphere and showed significant difference as compared to ISC group in total ($P < .0001$), basal ($P < .0001$), and apical ($P < .001$) dendrites. Cumulative frequency plot (Fig 6B) of total dendritic length showed a shift to the left in ISC group as compared to NC group indicating the dendritic atrophy. Hypertrophy can be seen in total dendritic length in ISC + CIMT group as the shift was found to the right of NC.

In the uninjured cortex (Fig 4B), the ISC group showed increase in total dendritic length as compared to NC. ISC + CIMT group had a significant increase in dendritic length as compared to NC ($P < .01$) in total dendritic length. Basal dendrites showed hypertrophy (notably at 40 μm-150 μm; supplementary Fig 5) in ISC + CIMT and in VC + CIMT. ISC + CIMT group showed a significant difference to NC ($P < .001$) and NC + CIMT ($P < .0001$). The VC + CIMT ($P < .01$) and ISC + CIMT ($P < .01$, mainly at radial distance 60 μm-120 μm from soma) had a significant difference as compared to NC + CIMT in apical dendritic length. The number of intersections of layer III pyramidal neuronal dendrite of both injured hemisphere and uninjured hemisphere (Supplementary Fig 7) showed similar trend as that of dendritic length. The neuronal

reconstruction of representative Golgi impregnated pyramidal neurons from injured and uninjured hemispheres in layer III has been depicted in Figure 4C.

Enhanced Dendritic Arborization in Pyramidal Neurons of Layer V Motor Cortex With CIMT After ISC Injury

CIMT had enhanced the dendritic arborization of layer V pyramidal neurons in both the hemispheres. In the injured cortex (Fig 5A), the total dendritic length did not alter significantly in the ISC group as compared to NC group. But the NC + CIMT ($P < .05$) and ISC + CIMT ($P < .05$) groups exhibited a significant increase in total dendritic length as compared to NC. In the uninjured cortex (Fig 5B), the ISC group showed a significant increase in total dendritic length in both basal (40 μm to 160 μm) and apical (50μm to 120μm) dendrites ($P < .05$) compared to NC group (Supplementary Fig 6). We observed a significant increase in total dendritic length in ISC + CIMT ($P < .001$) and VC + CIMT ($P < .05$) groups and Basal dendritic length ISC + CIMT ($P < .001$) and VC + CIMT ($P < .01$) compared to NC. The number of intersections of layer V pyramidal neuronal dendrite (Supplementary Fig 8) of both injured hemisphere and uninjured hemisphere showed similar trend as that of dendritic length. Figure 5C shows the neuronal reconstruction of representative Golgi impregnated pyramidal neurons from injured and uninjured hemispheres in layer V.

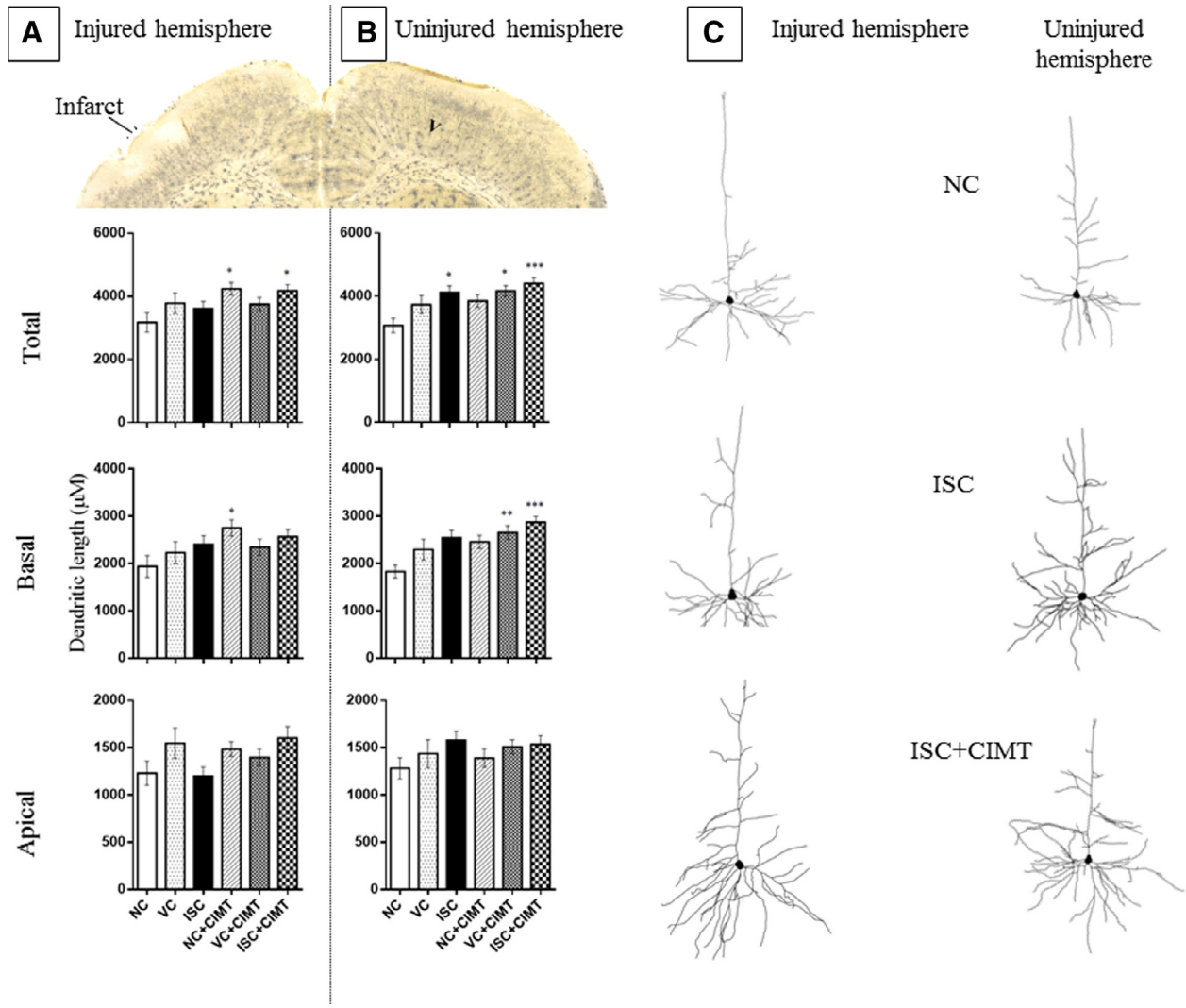

Figure 5. *Effect of CIMT after ISC stroke on dendritic length of layer V pyramidal neurons of (A) injured and (B) uninjured hemisphere. Significant changes were observed in both injured and uninjured hemisphere. (C) Neuronal reconstruction of representative Golgi-impregnated pyramidal neurons from injured and uninjured motor cortex layer V with NeuroLucida. Significant difference in the total dendritic length of NC + CIMT and ISC + CIMT groups was observed in comparison to NC (*P < 0.05; **P < 0.01; ***P < 0.001) in injured hemisphere.*

CIMT reduced the asymmetry in dendritic length across hemispheres caused by ISC injury

The NC group showed no significant difference (Mann-Whitney *U* test 2-tailed) for both basal and apical dendritic length in layer III and layer V across the hemispheres (Fig 6A), whereas the ISC group showed asymmetry in dendritic length in basal ($P < .0001$) and apical dendrites ($P < .0001$) in layer III pyramidal neurons. In Layer V only the apical dendrite showed significant difference in length ($P < .05$) across the hemispheres. CIMT intervention lead to a significant increase ($P < .05$) in layer III basal dendritic length. However, we did not observe any significant difference in the dendritic length in layer III apical dendrite, layer V basal, and apical dendrites across the hemispheres. The asymmetry caused by ISC stroke in the dendritic arborization across the injured and uninjured hemispheres was attenuated by CIMT. Figure 6C shows an overall summery of morphological study emphasizing on dendritic length in ISC and ISC + CIMT group.

Increased Axonal Density in Corpus Callosum With CIMT

Anterograde labeling with BDA revealed an increase in axonal density in the ISC + CIMT brain as compared to ISC brain. The qualitative study was done to observe the interaction between the hemispheres. The BDA was injected into the injured motor cortex and the anterograde tracing was found in the uninjured hemisphere (Fig 7A) especially in the corresponding motor cortex. Figure 7(B-E) shows the representative images of the axons crossing the middle of corpus callosum in ISC (Fig 7B, C) and ISC + CIMT (Fig 7D, E) rat brains. The axonal density of ISC + CIMT brain was much higher than the ISC brain indicating that the transcallosal interaction had increased with CIMT.

Discussion

ISC Stroke Model With Acute and Chronic Motor Deficits

Animal models that involve the MCA occlusion with ET-1 have been successful in producing the similar

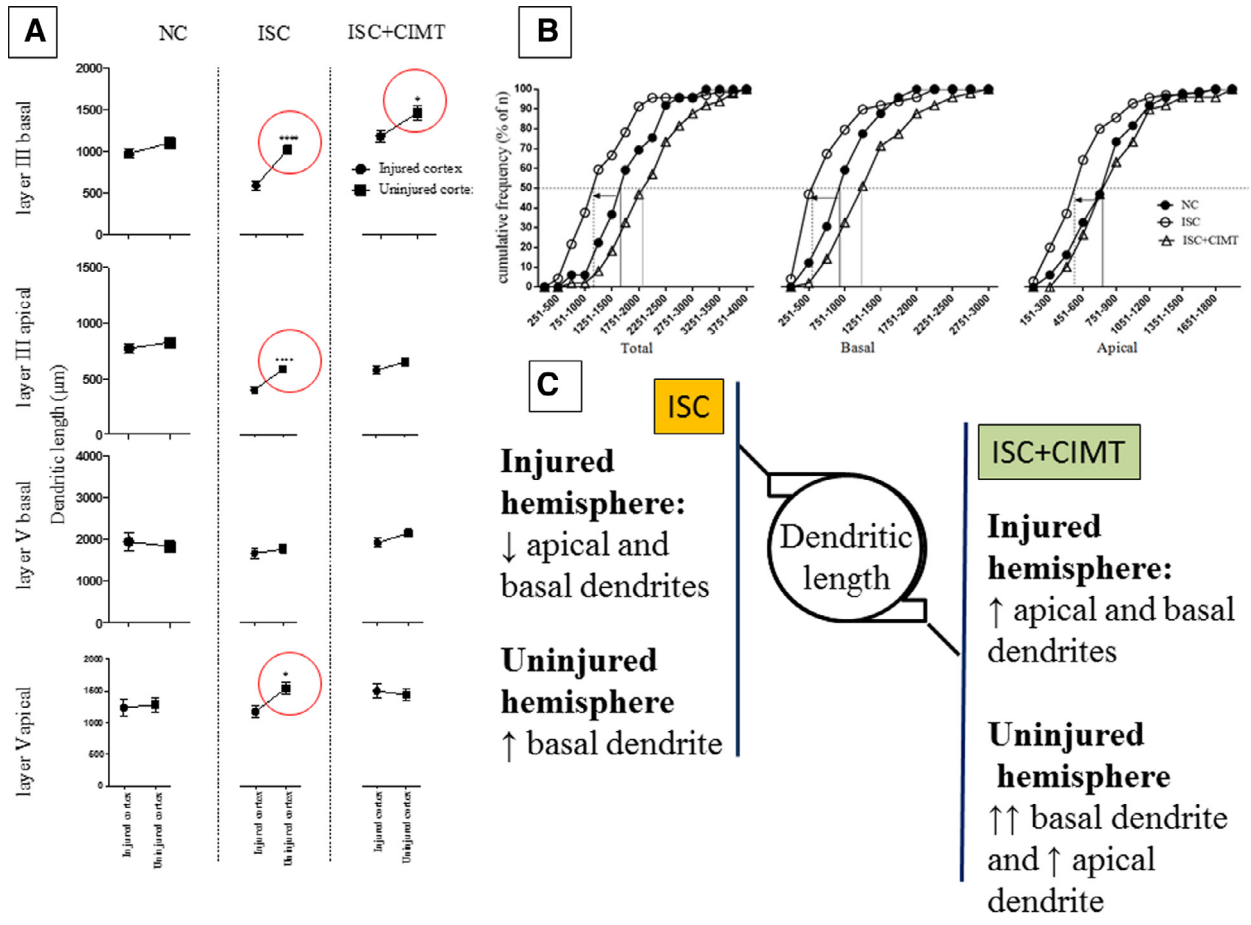

Figure 6. *(A) Comparisons between the injured and uninjured motor cortex in layer III and layer V apical and basal dendrites across the 3 groups—NC, ISC and ISC + CIMT. (B) Cumulative frequency plot of dendritic length of layer III pyramidal neurons of injured motor cortex. (C) Representative image of the overall summary of dendritic morphology of pyramidal neurons in layer III.*

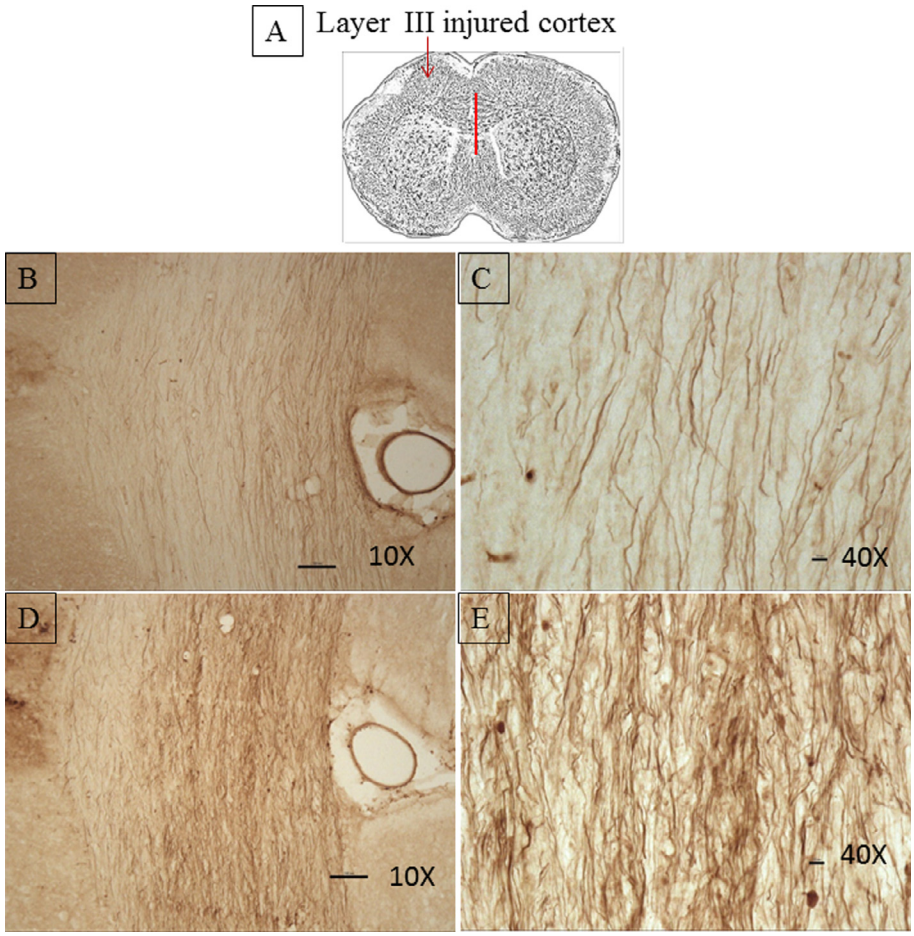

Figure 7. *Trascallosal axons in middle portion of corpus callosum (A) when traced with biotinylated dextran amine (BDA) anterograde labeling. Representative image of BDA positive labeled axons in ISC brain at (B) 10× (scale bar = 100 mm), (C) 40× (scale bar = 10 mm), (D) corresponding region in ISC + CIMT brain at 10× (scale bar = 100 mm), and (E) at 40× (scale bar = 10 mm).*

outcome in terms of pathophysiology and neurological deficits as that of humans. The MCA occlusion using ET-1 in rats has induced infarction in both striatum and sensorimotor cortex confirming the similarity with that of humans as that in earlier study thereby validating the ISC stroke rat model.[62] The ET-1 model is a transient model of ischemia that requires minimum surgical interventions,[63] leading to focal ischemia and reperfusion, thereby mimicking the human ISC stroke.[48,64] The low mortality rate observed with infusion of ET-1 to the near vicinity of MCA was an added factor for selecting the model for the study.[64] Twenty to thirty per cent of mortality was observed and it varied with climatic change. High air temperatures lead to increase in mortality rate which is similar to human ISC condition.[65-67]

The brain lesion induced by infusion of ET-1 was measured using TTC staining method.[68,69] The study found that the lesion size was relatively large with >30% of the total brain section, effecting striatum and sensorimotor cortex area—predominantly seen in the lateral part of the brain.

The effect of ISC lesion in the sensorimotor cortex on the neurological function was evaluated using Garcia score. The neurological deficits seen in these animals were not acute, but long lasting in nature. Since sensory feedback is essential during the execution of movement, the infarct in sensorimotor cortex might also add to the deficit in motor performance. Thus, the ET-1 model used in the present study was successful in mimicking the functional deficits observed in human stroke condition.[62]

ISC stroke altered the gait in these animals, with decreased stride length, not only in the affected limb but also in the remaining 3 limbs also. The reduction in the gait indicates the neural damage to motor cortex and striatum.[70,71] The present model of unilateral ISC stroke had asymmetry in the use of limbs during spontaneous vertical exploration in cylinder test which was observed throughout the study. This ipsilateral bias towards the unaffected limb was prominent in the ISC group even on day 17, indicating chronic deficit. The reach to grasp task is widely used to investigate the skilled forelimb movement in rodents and motor deficits involving the motor pathway. The impairment in fine movements while

testing with reach to grasp task showed deficit on day 5 and were consistent with the previous studies.[72,73] But as the days progress, the stroke rats tend to attain the task with altered movements,[74,75] such as using the unaffected limb or tongue to withdraw the pellet.

Regaining the Lost Functional Activity Following CIMT

Stroke rats subjected to CIMT were able to retrieve the pellet in a single attempt as indicated by increase in percentage of success after CIMT compared to control rats. Here we demonstrated not only the end measures in reach to grasp task, but also the quality of limb movements in reach to grasp task that was improved with CIMT. The number of abnormal movements had reduced significantly with the forelimb immobilization. Besides, the improved gross motor performance and asymmetry in spontaneous paw use was also had reduced with application of cast to unaffected limb. Rats were using both the paws simultaneously or independently to explore the walls of the cylinder. Refining of fine movement and improvement in spontaneous movement of the affected forelimb with CIMT directly indicate that CIMT helps in regaining the lost functional activity,[76] and was similar to human ISC studies.[77,78]

CIMT Induced Neural Plasticity in Motor Cortex Layer III and Layer V

Rostral forelimb area of the motor cortex[59,79] was selected due to its role in the neural control of digit flexion, wrist extension, and elbow flexion. These were important in execution of reach and grasp movements in animals. Our studies have shown distinct dendritic arborization pattern between layer III and layer V pyramidal neurons of the primary motor cortex. The differences in morphology consisted of a greater number of basal and apical dendrites in layer V pyramidal neurons when compared to layer III pyramidal neurons. We also found significant differences in dendritic architecture between basal and apical dendrites in terms of length and total number of dendritic branches.

Thus, we investigated the effect of ISC stroke on the dendritic morphology of pyramidal neurons of both layer III and layer V neurons. We found that ISC stroke has caused dendritic atrophy in layer III pyramidal neurons in the injured cortex. The decreased dendritic arborization in layer III pyramidal neurons of the injured cortex was observed in both basal and apical dendrites. However, the layer V of injured cortex did not show much dendritic atrophy as compared to controls. The apical dendrites were found to be affected more severely as compared to the basal dendrites in layer V in the injured cortex. On the other side, an increase in dendritic arborization was found in the uninjured cortex. This finding is similar to the previous studies in human subjects and rat model of ISC stroke[80,29] or fully in the stroke recovery after CIMT.

We found that ISC rats subjected to 7 days of CIMT showed increased dendritic length as well as dendritic branches in layer III pyramidal neurons. However, layer V pyramidal neurons did not show much difference in dendritic branches when compared to increased dendritic length after applying CIMT. As it is well-known that layer III of M1 receives the main input from M2, S1, S2 of same hemisphere[43,81] and from M1 of uninjured hemisphere.[82] Increase in dendritic arborization of layer III pyramidal neurons following CIMT in the injured cortex may represent the increased input into the injured cortex. The increase in the dendritic arborization in layer V pyramidal neurons of injured cortex with CIMT might indicate increase in the interlaminar interaction.[83] The horizontal connections from same hemisphere somatosensory cortex might have played an important role in functional recovery following CIMT. The uninjured cortex also showed enhanced dendritic arborization in both layer III and layer V.

An interesting finding in the present study was hypertrophy in the uninjured motor cortex after application of CIMT in ISC stroke group. The hypertrophy was found in both layer III and layer V especially in the basal dendrites of layer III pyramidal neurons. This raised an interesting question whether hypertrophy in the uninjured cortex could be due to the learned nonuse of the uninjured forelimb. Hence, we further analyzed both basal and apical dendrite length in the injured motor cortex with its corresponding uninjured region. The results showed no significant difference across the hemispheres in dendritic length for layer III and layer V apical and basal dendrites for NC group. The ISC group showed significant differences across hemispheres in both layer III and layer V apical and basal dendrites. The asymmetry in dendritic length was reduced across hemispheres in ISC + CIMT group for layer III and layer V. No significant differences were found in ISC + CIMT layer III apical, layer V apical, and basal dendritic length across the hemispheres. The present study for the first time has shown the reduction in the asymmetry of dendritic length with CIMT in the ISC injured brain. Increased dendritic arborization observed in both the hemispheres with application of the cast might have helped in reducing the asymmetry.

Modulation of Output From Uninjured Cortex

Increase in dendritic arborization in the uninjured cortex might also help in strengthening the uninjured motor output via pathways to the affected limb[28] in both ISC and ISC + CIMT groups. In that case ISC and ISC + CIMT should show similar improvement in the motor performance. Instead, the performance in the ISC group was poor as compared to ISC + CIMT group. The strengthening of ipsilateral pathways might help the patient to perform some essential movements. The increase in the alternative movements made by the rats can be read along

with the increase in the dendritic arborization in the uninjured cortex. The ISC + CIMT group not only had an increase in the dendritic length in the uninjured hemisphere but also enhanced interhemispheric interaction as compared to ISC group. This was revealed by the anterograde tracing with BDA. Transcallosal fibers arising from the injured motor cortex to the uninjured hemisphere[84] had found to be increased with forelimb immobilization. This was in support to the previous studies[25] on reduction in the interhemispheric inhibition with ISC insult. The reestablishment of transcallosal inhibition following CIMT after ISC stroke might have helped in modulating the output of uninjured cortex.

Thus, our morphological and behavioral study suggest that ET-1 infusion into the near vicinity of MCA resulted in substantial ISC lesions in both sensorimotor cortex and striatum showing characteristic motor and neurological deficits as that in humans. Further with the application of CIMT for 7 days with continuous training on skilled forelimb movement, we observed amelioration in the motor and neurological deficit observed in stroke rats. Quantitative measurement of dendritic remodeling along with behavioral assessment, demonstrate that CIMT can be one of the primary therapeutic strategies for the individuals with upper limb movement deficits.

Thus, we believe that our study would add evidences in understanding the modulation of cortical neurons resulting in the improvement of functional outcome with CIMT after ISC stroke. Also, the study provides further evidences in motor recovery which may help in translating the outcomes in clinical setup. Further attention is required to improve the understanding of underlying mechanisms following CIMT which might help in increasing the universal acceptability in adapting low-cost rehabilitative strategies like CIMT in human ISC stroke patients.

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Conflicts of Interest

The authors do not have any conflicts of interests.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jstrokecerebrovasdis.2019.02.028.

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