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Neurogenic Potential of *Clitoria ternatea* Aqueous Root Extract–A Basis for Enhancing Learning and Memory

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***Abstract*—**The neurogenic potential of many herbal extracts used in Indian medicine is hitherto unknown. Extracts derived from *Clitoria ternatea* Linn have been used in Indian Ayurvedic system of medicine as an ingredient of "Medhya rasayana", consumed for improving memory and longevity in humans and also in treatment of various neurological disorders. Our earlier experimental studies with oral intubation of *Clitoria ternatea* aqueous root extract (CTR) had shown significant enhancement of learning and memory in postnatal and young adult Wistar rats. The present study was designed to elucidate the *in vitro* effects of 200ng/ml of CTR on proliferation, differentiation and growth of anterior subventricular zone neural stem cells (aSVZ NSC's) derived from prenatal and postnatal rat pups. Results show significant increase in proliferation and growth of neurospheres and increase in the yield of differentiated neurons of aSVZ neural precursor cells (aSVZNPC's) at 7 days *in vitro* when treated with 200ng/ml of CTR as compared to age matched control. Results indicate that CTR has growth promoting neurogenic effect on aSVZ neural stem cells and their survival similar to neurotrophic factors like Survivin, Neuregulin 1, FGF-2, BDNF possibly the basis for enhanced learning and memory.

***Keywords*—**Anterior subventricular zone (aSVZ) neural stem cell, *Clitoria ternatea,* Learning and memory, Neurogenesis.

I. Introduction

NEUROGENESIS is an ongoing process in the anterior sub ventricular zone (aSVZ) and dentate gyrus (DG) of the hippocampus in mammals and humans from birth through adulthood into old age. It is well established that neurogenesis occurs throughout life and in adults is restricted to certain germinal niches of the brain like the subgranular zone and granule cell layer of the dentate gyrus in the hippocampus, the anterior subventricular zone lining the lateral ventricles, the olfactory bulb and the rostral migratory stream (RMS). The anterior subventricular zone (aSVZ) is a source of new neurons and glia throughout postnatal development into adulthood [1]-[4]. Neural progenitors of the SVZ are mitotically active and are restricted to different cellular compartments specialized for distinct cell lineages [5]-[7]. Neurogenesis in the aSVZ has been found to be up-regulated following stroke. It has been found that the newly born cells in the aSVZ migrate towards the site of the injury/ischemia rather than through the rostral migratory stream, perhaps helping in normal repair and restoration of the neural circuitry at the injured site [8]. A recent study by Coremans V et al [9] has shown that Survivin an inhibitor of apoptosis protein is normally expressed in neural precursor cells [NPC] of periventricular neurogenic regions in the embryo and postnatally gets restricted to proliferating and migrating NPCs in these two key neurogenic sites. Further, deletion of the *Survivin* gene causes mice brains to have fewer cortical inhibitory interneurons, contributing to profound deficits in learning and memory and enhanced sensitivity to seizures. Moreover a recent development on neurogenesis research states that layer 1 NPC's of neocortex form a source for adult neurogenesis in ischemia induced injury [10] suggesting that neural stem cells /or progenitor cells are maintained in the adult neocortex too and these can be activated by ischemia/ injury. Various studies indicate that factors such as Neuregulin 1, FGF-2, BDNF, HB-EGF positively enhance neurogenesis of stem and progenitor cells of various germinal niches in the brain and or neuronal growth [11]-[14]. Similar growth promoting factors in CTR may influence aSVZ NSC's grown in cultures.

Up-regulation of neurogenesis in germinal niches like the aSVZ with/without the influence of growth promoting factors is of prime interest since studies have shown that the newly born cells in the aSVZ migrate towards the site of ischemia /injury helping in repair and restoration of neural circuitry. Thus, it is of great interest to identify potential compounds /substances which help in positively enhancing neurogenesis and neuronal growth of aSVZ NSC'c, since they may be useful as therapeutics in stroke victims /patients with brain injury or to enhance learning and in memory in people with dementia.

Clitoria ternatea Linn (commonly called as butterfly pea), used in the Indian Ayurvedic system of medicine as an ingredient of "Medhya rasayana", a rejuvenating recipe containing concoction of herbal extracts is consumed for improving memory and longevity in humans and is also used for therapeutic treatment of various neurological disorders. In order to isolate the effects of a single herbal extract we designed studies using *the aqueous root extract of Clitoria ternatea alone***.** Our earlier experimental studies with oral intubation of *Clitoria ternatea* aqueous root extract to neonatal and young adult rats have shown that consumption of this extract significantly enhances spatial and passive avoidance learning and memory retention in both groups of rats as compared to age matched controls [15] and also

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significantly increases dendritic branching and intersections of CA3 pyramidal neurons of the hippocampus [16]. Further, our earlier studies have also shown that acetylcholine (ACh) content is significantly increased in the hippocampi of young adult rats orally intubated with *Clitoria ternatea* aqueous root extract as compared to ACh levels in hippocampi of age matched controls [17]. Our earlier studies have also shown that oral intubation of *Clitoria ternatea* aqueous root extract to neonatal and young adult rats, also significantly increases growth of neurons of the amygdala [18]. In addition histopathological study of the effect of oral intubation of *Clitoria ternatea* aqueous root extract to rats on their intestine, liver and kidney have shown *no toxic effect* in any of these organs (Data Unpublished)

Thus it is clear from our earlier studies that *Clitoria ternatea* aqueous root extract is non toxic and its oral intubation to rats have shown significant positive growth promoting effect on neurons at different sites in the brain and thereby functionally enhancing their learning and memory. But the influence of *Clitoria ternatea* aqueous root extract on neurogenesis and growth of newborn neural cells at any of the germinal niches of the brain, and its usefulness thereby as a therapeutic agent for treatment in stroke /brain ischemic injuries / neural disorders / for treatment of dementia / also for improvement of learning and memory is hitherto unknown. More specifically, the potential efficacy of the *aqueous root extract of Clitoria ternatea alone*, as a growth promoting neurogenic agent on aSVZ NSC's is unknown. We hypothesize that *Clitoria ternatea* aqueous root extract on aSVZ NSC's *in vitro* will enhance neurogenesis and will promote survival, proliferation, differentiation and growth of the newborn neural cells. Thus our present study was specifically designed to elucidate the influence of *Clitoria ternatea* aqueous root extract on aSVZ NSC's harvested from embryonic day 16[E16] rat brain and post natal day 3 [PND3] rat brain, seeded and grown in cultures *in vitro* for 7 days. Its effects thereof on neurogenesis and survival, proliferation, differentiation and growth of the newborn neural cells were tested.

II. Methods

A. Clitoria ternatea Aqueous Root Extraction

Clitoria ternatea aqueous root extract [CTR] processing was done as explained in [15].

B. Dissection of aSVZ Neural Stem Cells [aSVZ NSC's]

Fetuses [embryonic day 16/E16] from anaesthetized pregnant rats were first collected by caesarean section and Post natal day 3 (PND3) rat pups were anaesthetized by using halothane and their brains were collected by decapitation. Cerebral hemispheres of each brain were separated, by severing them from the brain stem. Slices of cerebral hemisphere containing anterior subventricular zone (aSVZ) tissue along with olfactory bulb were identified using a Nikon dissection microscope. The aSVZ tissue was carefully dissected out and collected into normal proliferation culture medium containing Dulbeco's modified Eagle's medium (DMEM), B-27 supplement without retinoic acid (1ml/50ml of medium;Gibco), EGF (20ng/ml;Sigma) and FGF-2 (20ng/ml;Peprotech), penicillin (100U/ml;Sigma) and Streptomycin (100ug/ml;Sigma) and Fungizone.

C. Proliferation of aSVZ NSCs

Following dissection, the aSVZ tissues were processed for dissociation and preparation of a single cell suspension using mechanical trituration. Using a fire-polished Pasteur pipette, tissue pieces were triturated 30-40 times in 2 ml of proliferation culture medium; the resulting cell suspension was diluted with 10 ml of fresh proliferation culture medium and spinned for 6-8 minutes. Cells were washed twice by re-suspension in fresh proliferation media. The final pellet obtained was re-suspended in 30µl of proliferation medium and viability of cells in single cell suspension was assessed using the Trypan- blue exclusion test. The cell suspension containing proliferation media and 400viable cells from both E16 and PND3 /well was plated onto the wells of one 96-well plate (Control group) containing 200µl of normal proliferation media/well and a second 96-well plate filled with 200µl of proliferation media containing 200ng/ml of CTR aqueous root extract (CTR-200)/well in each group. A fraction of plated aSVZ cells of both E16 and PND3 groups proliferated and formed neurospheres of aSVZ NSCs in all wells in both culture groups. Seven days after plating the number of neurospheres in each well were scored and the average number of neurospheres/well (i.e. #/400 plated cells) was compared between the two groups. Photomicrographic pictures of these neurospheres taken at 40X magnification were also used for quantification of the average size of neurospheres in the two groups-Control and CTR-200 of both E16 and PND3 cultures.

D. Differentiation of aSVZ NSCs from Neurospheres

Neurospheres derived individually from control and CTR aqueous root extract treated (200ng/ml) cultures of both E16 and PND3 were separately plated on to sterile and poly-L-lysine (0.05mg/ml) coated dishes (35mm Falcon dishes) and cultured with differentiation medium (1.5ml of medium/dish) containing Neurobasal medium (95.5ml/dl), B-27 nutrient mixture (with retinoic acid)-2ml/dl, L-glutamine (1.25 ml/dl) and Penicillin Streptomycin Fungizone (400µl/dl). Cultures were incubated in a humidified atmosphere of 5%CO_2 and 19 .8%O_2 at 37^{o}C for a period of 7 days *in vitro* (7DIV). The differentiation medium in the CTR group contained 200ng/ml of *Clitoria ternatea* (CTR) aqueous root extract. One-third of the differentiation medium was replaced every other day with fresh differentiation medium, with the CTR group containing the respective concentration of CTR (ie.200ng/ml). In both control and CTR root extract treated neurosphere differentiation cultures, neurosphere cells migrated away from the core of the neurosphere and differentiated. After 7 days of incubation in the differentiation medium, the cultures were fixed with 2% paraformaldehyde for 60 minutes. The 7 DIV cultures were processed for beta-III tubulin (TUJ-1; a marker of developing neurons) and glial fibrillary acidic protein (GFAP; a marker of astrocytes) dual immunofluorescence [19]. To visualize the nuclei of all cells the cultures were also

processed for DAPI fluorescence. Differentiation cultures of neurospheres in both the control and CTR200 treated groups were analyzed for yield of neurons from neurosphere cells. Neurospheres contain a central core of densely packed cells with a broader peripheral outskirt region surrounding the core showing dispersing/migrating neurons and glial cells. The central core of neurospheres was not used for counting of neurons since it contained densely packed cells including neurons. The peripheral outskirts of neurospheres containing clearly identifiable migrated neurons stained by TuJ1 and DAPI immunofluorescence were used for quantification. Neurospheres selected for quantification of % of neurons were similar in size and isolated from others, and neurons (TuJ1+ among DAPI cells) were counted from four randomly selected peripheral regions of 20 such isolated neurospheres/group.

E. Survival and Growth Profile of New Born Neurons

Another batch of 7 DIV PND3 aSVZ NS differentiation cultures of both the control and CTR200 treated groups, grown in 15mm diameter poly L-lysine coated dishes were also processed for beta- III tubulin (TuJ-1) and developed with Vector Grey, which marked the developing neurons and their dendritic growth profile. These were used for quantification of survival and growth of neurons. Survival analysis of these aSVZ neurons were quantified in both groups (control and CTR200), by counting of TuJ1 stained isolated neurons from 10 randomly selected peripheral outskirt field areas of neurospheres /culture dish in each group (each field is 25 mm^2) observed under 20X magnification using a 10X10 eyepiece grid.

Quantification and comparison of growth of neurons were also analyzed from the dendritic neurites of newly differentiated and migrated isolated neurons of the PND3 aSVZ neurospheres. Dendritic tracing was done (using Neurolucida) from randomly selected neurons that were isolated and migrated away from the neurospheres. These traced neurons from both controls and CTR200 treated groups were used for quantification and comparison of growth of neurons and dendritic cytoarchitecture. The identification of neurons was based on TuJ1 immunostaining of both soma and dendrites.

Statistical comparisons between the control and CTR 200 treated groups were done using two- tailed unpaired Students - *t* test and values of $p < 0.05$ was considered statistically significant.

III. Results

1. Proliferation of aSVZ NSCs: The number of E16 and PND3 neurospheres scored in each of the 20 wells and the average number of neurospheres/well (i.e #/400 plated cells) were compared between the control group and the group treated with 200ng/ml of *Clitoria ternatea* aqueous root extract. Comparison of the scores between the two groups reveal that the presence of CTR aqueous root extract in the culture medium significantly increases the yield of neurospheres from E16 aSVZ NSC's ($p<0.01$) [Fig.-1A] and PND3 aSVZ NSC's ($p<0.001$)[Fig-1B]. Additionally the average diameter of individual neurospheres from E16 and from PND3 at 7 DIV was significantly larger in CTR root extract treated cultures (E16 Control cultures, Mean± SEM= 164± 6.9µm;E16 CTR 200 treated cultures, 203± 5.8µm; $p<0.001$; Fig.2A and PND3 Control cultures, Mean± SEM= 195.37± 20.5µm;PND3 CTR 200 treated cultures, 361.39± 33.59µm; $p<0.0002$; Fig.2B) .

Since each of the neurospheres develop from a single NSC, increase in the number of neurospheres in CTR200 treated cultures reflect the positive influence of CTR aqueous root extract on proliferation and dispersion of individual NSC's to a significantly greater extent as compared to NSC's in control cultures. Likewise, the greater size of individual neurospheres in CTR200 treated cultures shows its influence on faster proliferation of the individual NSC's into neurospheres as compared to the development of NSC's into neurospheres in control cultures. Thus, it is evident that the presence of *Clitoria ternatea* aqueous root extract in aSVZ NSC cultures of both E16 and PND3 not only stimulates division and proliferation of individual NSC's into a large number to form neurospheres, it also positively enhances the proliferation of NSC's within individual neurospheres at a faster rate to form bigger spheres.

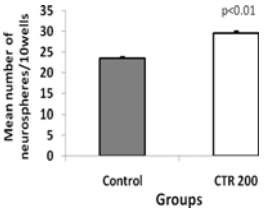

Fig. 1(a)

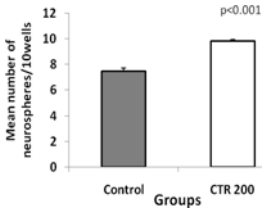

Fig .1(b)

Fig. 1(a) & 1(b) Proliferation of E16 1(a) and PND3 1(b) NSC's-Number of neurospheres at 7 DIV

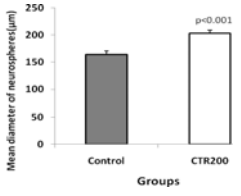

Fig . 2(a)

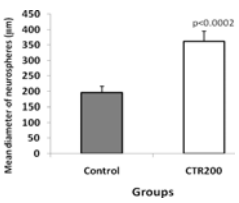

Fig. 2(b)

Fig. 2(a) & 2(b) - Proliferation of E16 2(a) and PND3 2(b)NSC's-Diameter of neurospheres at 7 DIV

2. Differentiation of aSVZ NSC's from Neurospheres: Differentiation cultures of E16 and PND3 aSVZ neurospheres in both the control and CTR200 treated groups were analyzed for yield of % of neurons among other cells migrated from neurospheres. Examination of the peripheral outskirts of E16 neurospheres (i.e the region of migrated cells) at 7DIV revealed significantly greater number of neurons ($p<0.0001$) in CTR200 root extract treated cultures than those of the control group. Quantification of the percentage of neurons revealed that 57.49% of all cells were neurons in CTR root extract treated cultures. In contrast, in control cultures, only 34.26% of all cells were neurons. Similarly examination of the peripheral outskirts of PND3 neurospheres at 7DIV revealed

significantly greater number of neurons ($p<0.0001$) in CTR200 root extract treated cultures than those of the control group. Quantification of the percentage of neurons revealed that 53.92% of all cells were neurons in CTR root extract treated cultures. In contrast, in control cultures, only 34.49% of all cells were neurons. Thus, it appears that the presence of CTR root extract considerably influences the differentiation of aSVZ NSC's into neuronal lineage and significantly increases the yield of neurons from both E16 and PND3 aSVZ neurospheres. This may also be due to positive influence of CTR root extract for significantly enhancing survival of newly formed neurons after differentiation, in cultures treated with CTR aqueous root extract as compared to those in the control cultures.

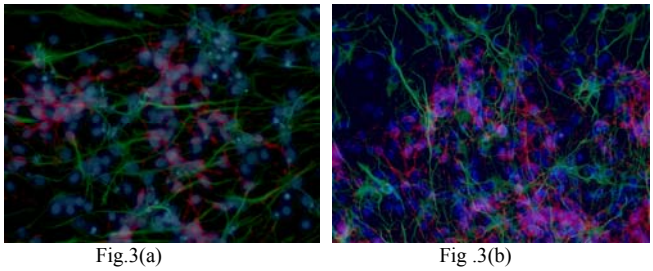

Fig.3(a) Fig .3(b)

Fig. 3(a) & 3(b)-Representative dual immunofluorescence images of differented TuJ1 positive neurons [red] and GFAP positive glial cells [green] migrated to the periphery of E16 NSC derived neurospheres from control [3(a)] and CTR200 groups[3(b)] at 7DIV. Nuclei of all neural cells are stained by DAPI [blue]

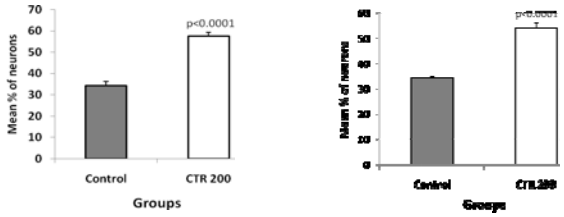

Fig. 4(a) Fig. 4(b)

Fig. 4(a) & 4(b)- Differentiation of E16 4(a) and PND3 4(b)NSC's into TuJ1 positive neurons at 7 DIV

3. Survival and Growth Profile of New Born Neurons

Differentiation cultures of PND3 aSVZ neurospheres in both the control and CTR200 treated groups were also analyzed and compared for survival of TuJ1 positive neurons that migrated from neurospheres at 7DIV. Results reveal significantly increased number (14.95±0.64) of neurons survive after differentiation and migration in cultures treated with CTR200 root extract as compared to those in control cultures (11.25±0.38; $p<0.003$).

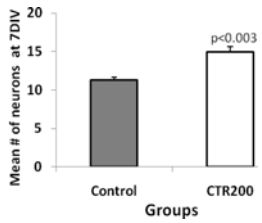

Fig: 5

Fig. 5 Survival of migrated TuJ1 positive neurons in PND3aSVZ cultures at 7DIV

In addition newly differentiated and migrated PND3aSVZ TuJ1positive Vector grey stained neurons [Fig 6A & 6B] were analyzed for their growth and development by analyzing their dendritic cytoarchitecture and results were quantified and compared between the control and CTR 200 treated cultures [Table-I].

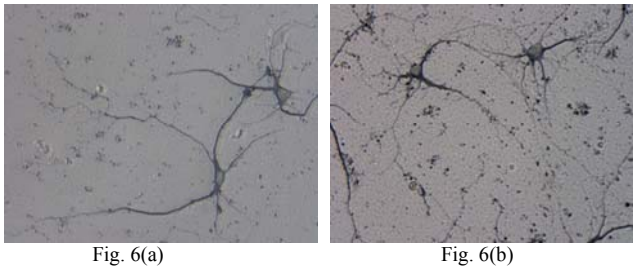

Fig. 6(a) Fig. 6(b)

Fig. 6(a) & 6(b) Representative photomicrographs of newly differentiated and migrated PND3 aSVZ TuJ1 positive Vector grey stained neurons from control [6(a)] and CTR200 [6(b)] groups at 7DIV

TABLE I
GROWTH PARAMETERS OF PND3 aSVZ DIFFERENTIATED TuJ1POSITIVE NEURONS

Groups	Area of soma(μm^2)	Dedritic profile			
		Number	Length (μm)	Nodes	Endings
Control	88.19±2.0	5.1±0.4	541.73±91.5	11.4±1.0	16.85±1.2
CTR200	96.05±2.9	7.15±0.7	1109.36±32.9	36.65±1.9	44.3±1.5
p <	NS	0.04	0.0001	0.0001	0.0001

Values represent Mean ±SE. NS= Not significant

IV. DISCUSSION

Neurogenesis occurs in isolated niches in the adult brain including the dentate gyrus (SGZ and GCL) in the hippocampus, the aSVZ lining the lateral ventricles, the olfactory bulb, the rostral migratory stream etc. Up-regulation of neurogenesis is seen following certain disorders like stroke, ischemia/hypoxia of the brain and also following exposure to excitotoxins [20],[8],[21],[22]. Studies also reveal that these newly born neurons and oligodendrocytes from regions like the aSVZ migrate towards the site of injury, indicating that these newly formed neural cells like type B cells from the SVZ may play a role in myelin repair in demyelinating lesions like multiple sclerosis and restoration of neural circuitry [23],[24]. Many studies provide ample evidence that up-regulation of neurogenesis occurring due to various factors stimulating the 'neurogenic reserve of hippocampal precursor cells' in the dentate gyrus and their plasticity enhances spatial learning and memory [25],[26] and a recent study [9] has also shown that lack of embryonic NPC survivin in the SVZ of mice results in fewer cortical inhibitory interneurons, contributing to enhanced sensitivity to seizures, and profound deficits in memory and learning.

Our present study shows the significant influence of *Clitoria ternatea* aqueous root extract on aSVZ neurogenesis

in vitro. CTR at a dose of 200ng/µl, significantly increases aSVZ NSC's to proliferate and form neurospheres and also enhances a faster rate of proliferation of the NSC's within neurospheres to form bigger neurospheres. In addition, CTR root extract considerably increases the yield of neurons from the aSVZ neurospheres, possibly due to its influence on inducing a greater number of the progeny of NSC's into a neuronal lineage. It also significantly enhances the survival and migration of these newly differentiated neurons from the aSVZ neurospheres in 7DIV cultures, reflecting its positive influence on neuronal survival and growth. The enhanced growth and development of the newly formed neuronal dendritic cytoarchitecture in CTR treated cultures indicates its positive influence on the growth of the newly formed neurons, which may provide the basis for functional plasticity, enhancement of learning and memory and also its ability to help in repair and restoration of functional neural circuitry in regions of brain affected by ischemia /injury.

The extent of neurogenesis in any of these germinal niches of the brain fluctuates depending on changes in the milieu of these niches. Studies show that increased levels of neurotrophic factors BDNF, FGF-2, EGF and others along with positive regulators like dietary restriction, exercise, environmental enrichment positively enhances neurogenesis of stem and progenitor cells and or neuronal growth of various germinal niches in the brain[27],[12],[14],[28]. Similar growth promoting factors in the aqueous root extract of *Clitoria ternatea* may have influenced the aSVZ NSC's grown in cultures.

Our earlier studies have shown that *Clitoria ternatea* aqueous root extract treatment *in vivo* in young adult and neonate rats enhances both spatial learning ability and retention of learned tasks [15] and these rats showed a significant increase in dendritic growth profile of CA3 pyramidal neurons [16] and an increase in hippocampal acetylcholine content [17]. However, the mechanism/s by which CTR may bring about this enhancement in learning and memory was hitherto unknown. Our present *in vitro* study shows that *Clitoria ternatea* aqueous root extract positively influences hippocampal and SVZ neurogenesis, perhaps forming the basis for the enhanced spatial learning and memory observed in our earlier *in vivo* study.

V. CONCLUSION

Clitoria ternatea aqueous root extract has the potential of being a neurogenic growth promoter and may be envisaged as a therapeutic compound to step up aSVZ and hippocampal neurogenesis and increase in survival, growth and development of the new born neurons, critical for repair and restoration of the normal neural circuitry in various disorders like stroke, ischemia of brain or injury to the brain /spinal cord.CTR may also be used as a neurogenic growth promoting factor to enhance neural stem cell growth and differentiation *in vitro* for strategic therapeutic neural stem cell transplantations for repair and regeneration in cases of brain /spinal cord injuries. This extract may also be envisaged for use as a memory enhancer in dementia/any disorder related to loss of learning ability and/disorders of memory as in Alzheimer's disease.

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