Online ISSN: 2249-4626
Print ISSN: 0975-5896

Global Journal

of Science Frontier Research: G

Bio-Tech & Genetics



Processing of Catechol

DNA Barcoding Identification

Highlights

Autosomal DNA Markers

Intermediate of Aromatic Compound

Discovering Thoughts, Inventing Future

VOLUME 15 ISSUE 1 VERSION 1.0

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Volume 15 Issue 36 was originally issued as G1

Global Journal of Science Frontier Research: G
Bio-Tech & Genetics

Volume 15 Issue 36
Originally issued as G1

Global Journal of Science Frontier Research: G

Bio-Tech & Genetics

Volume 15 Issue 1 (Ver. 1.0)

Open Association of Research Society

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Global Journal of Science Frontier Research: G
Bio-Tech & Genetics
Volume 15 Issue 1 Version 1.0 Year 2015
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN:2249-4626&Print ISSN:0975-5896

DNA Barcoding, Identification and Validation of the Genus *Phyllanthus* in Nigeria using Plastid rbcL and matK Genetic Markers

By Daniel Azubuike Awomukwu, Bio Louis Nyananyo, Chiedozie Joel Uka, Paula Spies & Bulelani Londoloza Sizani

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Abstract- DNA of five species of *Phyllanthus* in Nigeria namely, *P. amarus* Schum and Thonn, *P. urinaria* Linn., *P. odontadenius* Mull-Arg., *P. niruroides* Mull-Arg. and *P. muellerianus* (O. Ktze) Excel belonging to the family of Phyllanthaceae were extracted, purified, PCR amplified and sequenced using plastid Ribulose -1,5 bisphosphate carboxylase large chain (rbcL a) and Maturase K (matK) genetic marker to identify unknown *Phyllanthus* species. The plastid region revealed that the *Phyllanthus* species were able to be amplified optimally for sequencing. The results of the nucleotide sequences were further compared on Basic Local Alignment Sequence Tool (BLAST) on Gen Bank and BoldSystems for validation. Results revealed that the closely related species, *P. niruroides* Mull_Arg. and *P. odontadenius* Mull-Arg. had no DNA record to separate them on both GenBank and BoldSystems using both rbcL and matK gene regions while *P. amarus* Schum and Thonn and *P. urinaria* Linn. were clearly compatible with other works. *P. muellerianus* (O. Ktze) Excel was only compatible with other works using matK gene region but none for rbcL.

Keywords: Phyllanthus species, DNA extraction, PCR amplification, nucleotide sequences, BLAST, Bold Systems, rbcL, matk, Barcoding.

GJSFR-G Classification : FOR Code: 069999



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DNA Barcoding, Identification and Validation of the Genus *Phyllanthus* in Nigeria using Plastid rbcL and matK Genetic Markers

Daniel Azubuike Awomukwu [α], Bio Louis Nyananyo [σ], Chiedozie Joel Uka [ρ], Paula Spies [ω] & Bulelani Londoloza Sizani [¥]

Abstract- DNA of five species of *Phyllanthus* in Nigeria namely, *P. amarus* Schum and Thonn, *P. urinaria* Linn., *P. odontadenius* Mull-Arg., *P. niruroides* Mull-Arg. and *P. muellerianus* (O. Ktze) Excel belonging to the family of Phyllanthaceae were extracted, purified, PCR amplified and sequenced using plastid Ribulose -1,5 bisphosphate carboxylase large chain (rbcL a) and Maturase K (matK) genetic marker to identify unknown *Phyllanthus* species. The plastid region revealed that the *Phyllanthus* species were able to be amplified optimally for sequencing. The results of the nucleotide sequences were further compared on Basic Local Alignment Sequence Tool (BLAST) on GenBank and BoldSystems for validation. Results revealed that the closely related species, *P. niruroides* Mull_Arg. and *P. odontadenius* Mull-Arg. had no DNA record to separate them on both GenBank and BoldSystems using both rbcL and matK gene regions while *P. amarus* Schum and Thonn and *P. urinaria* Linn. were clearly compatible with other works. *P. muellerianus* (O. Ktze) Excel was only compatible with other works using matK gene region but none for rbcL. BLAST validation also revealed that *P. amarus* is synonymous with *P. niruri* due to overall similarity they share on both rbcL and matK genome and needs to be subsumed. Results of the nucleotide sequences and fragment analysis were published on BoldSystems for barcoding as a standard coding marker translation matrix.

Keywords: Phyllanthus species, DNA extraction, PCR amplification, nucleotide sequences, BLAST, BoldSystems, rbcL, matK, Barcoding.

I. Introduction

One of the most reliable methods for identification of medicinal plants involves morphological and genetic analysis. Molecular techniques have been also introduced for DNA fingerprinting (Sucher and Charles, 2008). Analysis of the DNA that is present in all organisms is a suitable method for identifying plant materials because the genetic composition is unique for each individual organism. DNA extracted from the leaves, stems or roots of plants all carry the same genetic information without being affected by physiological conditions and environmental factors. Polymerase Chain Reaction (PCR) in combination with Sequencing and DNA barcoding has been widely used for DNA fingerprinting (Weisling *et al*., 2005). Species-species regions in nuclear DNA, mitochondrial DNA and chloroplast DNA have been used for identification of individual species. DNA fingerprinting is an important tool for molecular characterization of various groups of plants. It offers a faster and more precise way of determining relationships among closely related species than that of morphological investigation (Rahman, 2007). This is because morphological characteristics are subject to environmental influence and extensive studies of mature plants are often necessary for taxonomic classification.

The first proposed DNA-barcoding regions for universal plant DNA-barcoding suggested by the PWG, were the multicopy nuclear Internal Transcribed Spacer (ITS), the rbcLa subunit and matK (Stoeckle *et al*., 2004). Since then, several barcoding regions have been investigated, tested and proposed for different groups. Gene regions that are popular in phylogenetic studies have been investigated for possible candidate regions to be used in barcoding. Loci that are popular in plant systematics are rbcL, the trnL-F intergenic spacer, matK, ndhF and atpB. Two of these regions, rbcL and atpB, are used in phylogenetic studies to distinguish at genus level and above. Even though the characteristic of a suitable barcoding region is that there should be distinction at the species level, rbcL and atpB have been considered as barcoding regions (Blaxter, 2004). The regions matK and ndhF have enough variation to be used in phylogeny on interspecific level, but unfortunately only when the sequenced length is more than 1000bp does it provide enough variation for discrimination (Kress *et al*., 2005). The CBOL plant working group proposed the use of rbcL and matK as universal plant barcoding regions

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(CBOL Plant Working Group, 2009). The SciVerse Scopus bibliography database (accessed on October 2012) have 283 citations to this article and the common conclusion of many of these studies is that a universal barcode system can still not be agreed upon due to lack of universality, sequence quality and lack of discriminatory power (CBOL Plant Working Group, 2009).

The molecular data of *Phyllanthus*, the third largest genus in the family, has received considerable attention as an important character in inferring systematic relationships (Wurdack *et al*., 2004; Samuel *et al*., 2005; Kathriarachchi *et al*., 2005). The potential to apply barcoding in plant taxonomy were first explored during an exploratory workshop in 2003 (held at the "Cold Spring Harbor Banbury Conference Center" from 9 – 12 March; accessed on http://www.barcodeoflife.org/content/about/what-cbol) and it was predicted that barcoding will in future be utilized in species identification, conservation biology and mapping the extent of species by linking maps to barcodes. It was also predicted that the cost of barcoding a sample would decrease to such a degree that it would be affordable to be used by science teachers and "backyard naturalists" (Stoeckle *et al*., 2003). A DNA-barcode is "a short DNA-sequence that identifies a species" (Stoeckle *et al*., 2003), by comparing the sequence of an unknown specimen to barcodes in a sequence database of known species (Kress & Erickson, 2007). The main use of these sequences is for identification and not for phylogenetic reconstruction (Kress & Erickson, 2007) or as only criterion in describing new species (Stoeckle *et al*., 2003). It differs from molecular phylogeny in that the main goal is not to determine patterns of relationship but to identify an unknown sample in terms of a preexisting classification.

Phyllanthus has a remarkable diversity of growth forms (annual and perennial herbaceous, arborescent, climbing, floating aquatic, pachycaulous, and phyllocladous), floral morphology (Bancilhon, 1971), and chromosome numbers (Webster and Ellis, 1962). The diversity of pollen types (Ko¨ hler, 1965, 1967; Punt, 1967, 1987; Webster and Carpenter, 2002; Sagun and Van der Ham, 2003) rivals that of any genus of flowering plants. The vast majority of *Phyllanthus* species, however, share a distinctive vegetative specialization known as ''phyllanthoid branching'' (Webster, 1956) with leaves on the main axes reduced to scales called ''cataphylls'' and those on lateral (plagiotropic), deciduous, floriferous axes developing normally.

Phyllanthus has a long history of use in tropical countries in indigenous medicine for the treatment of liver ailment. They were examined during several researches. The pharmacognistic importance of some of these *Phyllanthus* species found in Nigeria has been elucidated by Awomukwu *et al*. (2014). These medicinal plants have been underutilized in orthodox medicine but have confirmed to be used worldwide in the pharmaceutical, food, cosmetics and perfume industries. Different authors in different groups have studied the taxonomic significance and scientific implication of the morphological, anatomical and epidermal features of *Phyllanthus* species in Nigeria. They include Edeoga *et al*. (2007) and Uka *et al*. (2014). Awomukwu *et al*. (2015) has successfully delineated the indigenous species occurring in Nigeria based on its nuclear ribosomal Internal Transcribed Spacer (ITS 4-5) genetic marker in which the DNA molecular data sequences were able to identify, validate and classify the various species. None of such works have been done on barcoding using the concatenation of rbcL and matK gene regions. Series of documented descriptions of the morphological, anatomical, ethnobotanical and phytochemical characteristics of *Phyllanthus* exist; there is confusion in recognizing individual species of these plants in Nigeria. The aim of this work is to provide reliable genetic information in order to aid easy recognition and to authentically barcode the *Phyllanthus* species for pharmacognistic research with reference to modern day genetic search tools.

II. Materials and Methods

a) Collection of plant materials

Mature plants of the five species *P. amarus, P. urinaria, P. niruroides, P. odontadenius* and *P. muellerianus.* were collected from different locations of Nigeria (bounding box coordinates: upper left – 6.3333, 7; lower right – 4.75, 6.8333) by various investigators as in Table 1. Only healthy, fresh and succulent parts of the plants were collected. The five specimens were identified and authenticated at the Herbaria of the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State and the Department of Botany, Nnamdi Azikiwe University, Awka, Anambra State. Herbarium specimens were also studied at the various institutions as well making reference to the Flora of West Tropical Africa by Hutchinson and Dalziel (1963). The accessions were deposited at the Herbarium of the Department of Plant Science and Biotechnology, University of Port Harcourt, Port Harcourt, Rivers State, Nigeria with their sample and process identity numbers for further research. The same specimen records were also submitted on public data portal at Boldsystems. Further laboratory analysis was carried out at the Molecular Genetic Lab, University of the Free States, Bloemfontein, South Africa.

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Table 1 : Collection sites, identity numbers and collection dates of the five *Phyllanthus* species studied

SPECIES	SITE OF COLLECTION	CORDINATE/ ELEVATION	SAMPLE ID	PROCESS ID	DATE OF COLLECTION
P. amarus	Along the school fence, Abia State Polytechnic, Aba.	N5.42; E6.33; 25.0m	AWOM UPH PA 010	PHSN 001-14	April 15, 2014
P. urinaria	Field around National Root Crop Research Institute, Umudike.	N4.75; E6.83; 20.0m	AWOM UPH PN 050	PHSN 003-14	April 14, 2014
P. odontadenius	Road side along National Root Crop Research Institute, Umuahia.	N5.42; E7.50; 25.0m	AWOM UPH PO 040	PHSN 006-14	April 15, 2014
P. niruroides	Science Village, Nnamdi Azikiwe University, Awka.	N6.33; E7.00; 22.0m	AWOM UPH PU 030	PHSN 005-14	April 15, 2014
P. muellerianus	Near the Herbarium Building, Nnamdi Azikiwe University, Awka.	N6.33; E7.00; 23.0m	AWOM UPH PM 020	PHSN 002-14	April 15, 2014

b) DNA extraction and PCR amplification

Total genomic DNAs were extracted from young, dry leaves of *Phyllanthus* specimens using the CTAB method by Doyle & Doyle (1987), because of high amount of polysaccharides present; the CTAB concentration was adapted to 3% (Doyle & Doyle 1990). The DNA pellet was then diluted into 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). An additional phenol/chloroform purification step was performed to remove proteins and potentially interfering secondary metabolites as follows: 150 µl of TE was added to bring the initial volume to 200 µl. Equal volume of phenol was then added, vortex gently and spin for 2 min at 13 000 rpm at room temperature. It was followed by addition of 100 µl of Sevag (chloroform-isoamyl alcohol 96:4), centrifugation at 13 000 rpm at room temperature of the collected aqueous steps (repeated twice). DNA was precipitated by incubating with 2/3 100% EtOH-3M NaOAc (25:1) at -20°C for 24 hours, then centrifuged for 2 min at 13 000 rpm at 4°C. The DNA pellet was washed with 70% EtOH, centrifuged at 13 000 rpm for 5 min, air dried in the dark and finally re-suspend in 50 µl TE.

The plastid regions, rbcL and matK (Table 2) were amplified using either direct PCR (Finnzymes Direct PCR kit) or iProof™ High-Fidelity DNA Polymerase (Bio-Rad Laboratories, CA) according to the manufacturer's protocol. iProof GC buffer (Bio-Rad) was used when nuclear phylogenetic gene amplification with HF buffer (Bio-Rad) did not provide satisfactory results. A finally concentration of 5% DMSO was added in 20 µl PCR reaction. In few cases direct PCR was done on dry leaves or on fresh leaves stored at -20°C.

The PCR amplifications were performed using a G storm PCR system 9700 (Perkin-Elmer) with the following thermal cycle conditions unless stated otherwise: DNA was initially denatured at 98°C for 5 minutes followed by 40 cycles at 98°C for 5 seconds, primer annealing at 55.8°C (for rbcL) and 55.5°C (for matK) for 5 seconds and elongation at 72°C for 20 seconds with a final 1 minute elongation at 72°C. The PCR fragment lengths were determined on a 1% agarose gel (see plate 2 and 3)

c) DNA Sequencing

PCR products were sequenced directly after 1:5 diluting with dH_2O. Amplified regions were sequenced in both directions with an automated sequencer 3730 Genetic Analyser Applied Biosystems v1.1/3.1 Cycle Sequencing Kit, according to the protocol provided with few modifications. Briefly: the component and volumes for the sequencing PCR reactions were: 1 µl of 5x sequencing buffer, 0.5 µl premix (Applied Biosystems), 3 µl of 10 µM primer, 3 µl dH_2O, 5% DMSO and 2 µl PCR product were used. Cycle sequencing steps were as follows: initial denaturation at 96°C for 1 min, following by 25x cycles of 96°C for 10 seconds (with a ramp seed of $3°Cs^{-1}$), 48°C for 15 seconds, 60°C for 4 min; 72°C for 1 min.

Table 2 : Nucleotide sequence data of the primers used for the amplification of the regions.

Region	Primer name	Primer sequence 5'-3'	Tm (°C)	MW	GC(%)	Length
matK	matK-472F	CCCRTYCATCTGGAAATCTTG	57.8	7583.5	42.86	21
	matK-1248R	GCTRTRATAATGAGAAAGATT	52.5	8033.3	23.81	21
rbcL	rbcLa-F	ATGTCACCACAAACAGAGACT	57.2	7950.3	42.86	21
	rbcLa-R	GTAAAATGTAGTCCACCRCG	52.8	6087.0	40.00	20

d) Cleanup

Extension products were adjusted to 20 µl by adding 10 µl of dH_2O, precipitated with 5 µl of 125 mM EDTA, and 60 µl of absolute EtOH for 15 min and centrifuged for 15 min at 20 000 g. For pellet purification, 60 µl of 70% EtOH was added after

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removal of supernatant, centrifuged for 5 min at 20 000 g at 4°C. Tubes were then dried at 55°C for 5 min.

e) Sequence Alignment and Data Analysis

The data sequences of the rbcL and matK regions were aligned respectively, followed by manual adjustment and trimming at the ambiguous ends. The software programme Geneious Pro 7.0.4 (Biomatters, Ltd., http://www.geneious.com) with the default alignment parameters was used to view, assemble and edit the sequence trace files. Consensus sequences were aligned with the MUSCLE plug-in in Geneious R7. The post-trimmed lengths were at least 80% of the original read length. Sequences which covered more than 70% overlap between the forward and reverse sequences were considered. Other statistical values for each gene region such as the composition, GC content, % pairwise residue, % identical alignment, the minimum, maximum and mean lengths for the sequences as well as the pairwise number were also obtained in Geneious R7. Consensus sequences were compared on GenBank nucleotide Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov/Blast.cgi) and Boldsystems (http://www.boldsystems.org) for species identity validation.

f) DNA Barcoding Method

The project was registered under the workbench portal on the website (http://www.boldsystems.org) which is the software for DNA barcoding. Information on the title, code, description, campaign and markers were provided. This required integration of a field information management system (FIMS), laboratory information management system (LIMS), sequence analysis tools, workflow tracking to connect field data and laboratory data, database submission tools and pipeline automation for scaling up to eco-system scale projects. The genetic information were obtained from the concatenation of the rbcL and matK chloroplast genes. Then after, the details of the specimen data (such as species name, voucher data, collection record, identifier and primer information), images, sequences and trace files will be uploaded by following the formal submission guidelines and instructions on the website. The deposited sequences will automatically be tested for incongruence by the website software and validated by the database administrators before publication.

III. Results

Fig 1. shows the map of the various areas in which the samples were collected in Nigeria. From the information below, it could be gathered southern zone of Nigeria have abundance of these species. Virtually, all the states within southern zones of the country served as a sample area for the collection of the species. This does not actually apply that the species are endemic to this area or that they do not exist in other areas of the country. The map presented herein shows the states in the country that fall within the bounding box coordinates: upper left – 6.3333, 7; lower right – 4.75, 6.8333. The states included: Abia, Akwa Ibom, Anambra, Bayelsa, Cross River, Ebonyi, Enugu, Imo and Rivers. Mature plants of these five species, *P. amarus, P. urinaria, P. niruroides, P. odontadenius* and *P. muellerianus.* exist within these sites and location as mentioned in Table 1 above.

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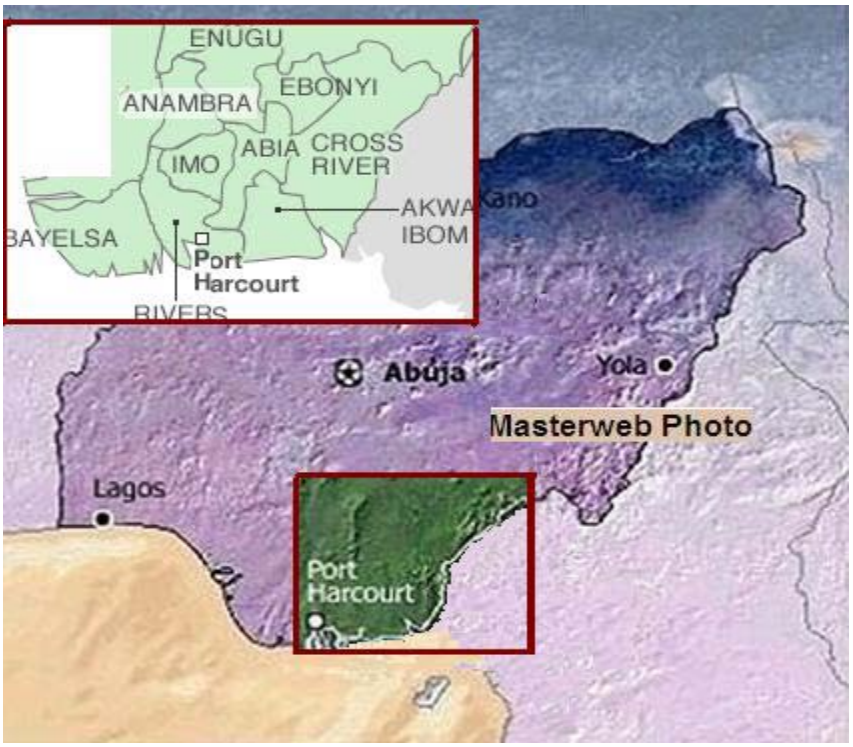


Figure 1 : Map of Nigeria showing the collection sites of the species of *Phyllanthus* used in the study





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2b.i


2b.ii


2c.i


2c.ii


2d.i


2d.ii

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Figure 2 : Photographs of the morphology of the genus *Phyllanthus* studied.

(a.i) *P. amarus*, showing the dorsal surface of the herb and the elliptic-oblong shape of the leaves.

(a.ii) *P. amarus*, exposing the greenish fruits and the pentatepalous flowers on the ventral surface of the leaves.

(b.i) *P. urinaria*, showing the dorsal surface of the herb and the mucronate leaf apices .

(b.ii) *P. urinaria*, exposing the reddish-green friuts, stipules and buds on the ventral surface of the leaves.

(c.i) *P. odontadenius*, showing the dorsal surface of the herb and the oblong shape of the leaves.

(c.ii) *P. odontadenius*, exposing the whitish-green tepals and reddish laterally free stipules.

(d.i) *P. niruroides*, showing the dorsal surface of the herb and the mucronate leaf apices.

(d.ii) *P. niruroides*, exposing the greenish fruits and tepals and reddish laterally free stipules.

(e.i) *P. muellerianus*, showing the dorsal surface of the shrub branch and the ovate-elliptic shape of the leaves.

(e.ii) *P. muellerianus*, exposing the spiny stipules of the leaves and thorny branches.

In this study, DNA quality was assayed by gel electrophoresis and intense bands were seen on 0.8% agarose. Only duplicate samples of each species were selected for sequencing. The thick band showed the PCR amplified rbcL (Plate 1) and matK (plate 2) regions which ranged from 500bp – 600bp in the respective samples. The leaf materials used were mainly dry leaves stored in silica gel. The samples contained both old and young leaves. The older and harder leaves had a higher level of secondary compounds which could act as PCR inhibitory components. The young leaves of *Phyllanthus* yielded a better quality DNA than the older, outer leaves of the plants.

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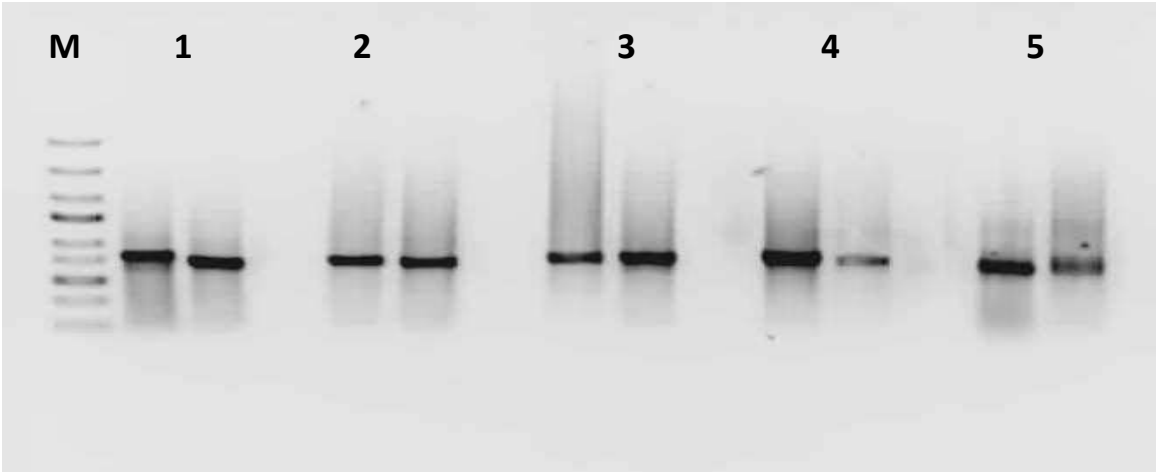


Plate 1 : PCR profile of the amplified rbcL gene region of the leaf of the five *Phyllanthus* species investigated. DNA fragment size range from 500bp – 600bp. Each species is represented by a duplicate of the samples. 1 – *P. amarus*, 2 – *P. muellerianus*, 3 – *P. niruroides*, 4 – *P. odontadenius*, 5 – *P. urinaria*

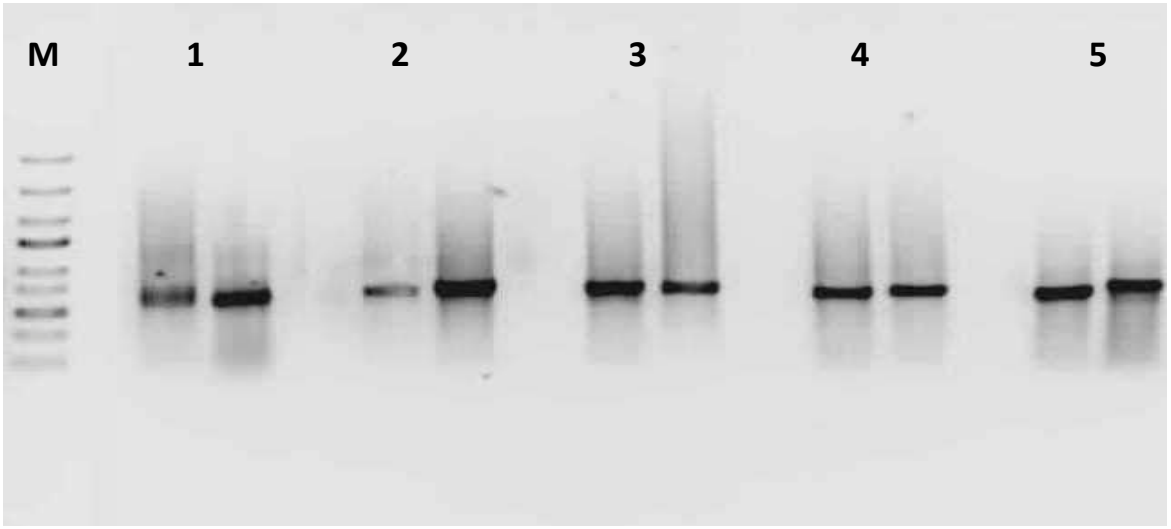


Plate 2 : PCR profile of the amplified matK gene region of the leaf of the five *Phyllanthus* species investigated. DNA fragment size range from 500bp – 600bp. Each species is represented by a duplicate of the samples. 1 – *P. amarus*, 2 – *P. muellerianus*, 3 – *P. niruroides*, 4 – *P. odontadenius*, 5 – *P. urinaria*

When visualized on an agarose gel, the DNA from the young leaves had less or no smears. Even by adapting the extraction protocol by adding recommended chemicals (such as PVP and SDS) at different stages in the process, the secondary compounds (e.g. polysaccharides and polyphenolic compounds) could not be removed completely. The polysaccharides co-precipitate with the DNA (Kumari *et al.*, 2012), resulting in a viscous solution. Some leaf samples, where the direct PCR approach was followed, seemed to contain PCR inhibitory components, and were excluded from the study after 3-4 amplification attempts. For future studies on *Phyllanthus* it is suggested that the young leaves are used for DNA extraction, or alternatively that the method of Kumari *et al.* (2012) be implemented as an extraction protocol.

The aligned rbcL matrix consisted of 475 base pairs (bp), 95.7% pairwise residue, 90.1% identical alignment, 475 bp maximum sequence and 475 bp minimum sequences while the aligned matK matrix consisted of 481 base pairs (bp), 88.3% pairwise residue, 74.4% identical alignment, 469 bp maximum sequences and 360 bp minimum sequences (Table 3).

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Table 3 : Matrix and statistics of the rbcL and matK data sets in the study

Sampling Information	rbcL	matK	Description
Sequence Length (bp)	475	481	Residue length of sequence.
Number of species sequences	5	5	Number of sequences in an alignment or set of sequences.
Pairwise residue (%)	95.7	88.3	Percentage of parwise residues that is identical in the alignment, including gap vs non- gap residue, but excluding gap vs gap residue.
Identical alignment (%)	90.1	74.4	Percentage of columns in the alignment for which all sequences are identical.
Maximum sequence (bp)	475	469	Maximum length of non-reference sequences in a sequence list of or alignment.
Minimum sequence (bp)	475	360	Minimum length of non-reference sequences in sequence list or alignment.

The information on the composition of the various nucleotide bases of the sequences obtained from the samples as amplified from the primer gene markers were analyzed and summarized in the Table 4 and Table 5. The total number of sequence length per species was also recorded. The record was generated from BoldSystems during the analysis and submission.

Table 4 : Summary of the composition of the nucleotide bases from rbcL sequence data

TAXA	NUCLEOTIDE COMPOSITION	TOTAL SEQUENCE LENGTH (bp)
P. amarus	A (169), G (117), C (133), T (154)	573
P. muellerianus	A (150), G (141), C (132), T (166)	589
P. urinaria	A (169), G (118), C (130), T (156)	573
P. odontadenius	A (156), G (133), C (117), T (169)	546
P. niruroides	A (169), G (118), C (131), T (152)	570

Table 5 : Summary of the composition of the nucleotide bases from matK sequence data

TAXA	NUCLEOTIDE COMPOSITION	TOTAL SEQUENCE LENGTH (bp)
P. amarus	A (257), G (118), C (98), T (200)	673
P. muellerianus	A (163), G (88), C (90), T (203)	544
P. urinaria	A (151), G (88), C (93), T (186)	518
P. odontadenius	A (178), G (93), C (86), T (172)	529
P. niruroides	A (153), G (88), C (81), T (188)	518

During the research in BOLD, the different sequences were compared and the differences were represented in a form that resembles barcodes in which each colour represents one of the nucleotide bases of DNA [Adenine (A), Thymine (T), Cytosine (C) or Guanine (G)]. Like a barcode for products in a shop, each species of the *Phyllanthus* investigated had unique nucleotide barcoding pattern in BOLD and were identifiable from this unique pattern. Other *Phyllanthus* barcodes submitted on BOLD were compared with our accessions and only *P. amarus* and *P. urinaria* had previous records of other authors. *P. muellerianus, P. niruroides* and *P. odontadenius* had no previous submitted sequence records on BOLD and therefore were the first time to be registered and submitted on the website (http://www.boldsystems.org). Table 6 and 7 displays the information on the link of the accessions published on BOLD with the illustrative barcodes being developed in the linked website for each taxon.

Table 6 : Information and links of the *Phyllanthus* species accessions submitted on BOLD Systems for rbcL gene region

TAXA	PROCESS ID	SEQUENCE ID	LINK/REFERENCE
P. amarus	PHSN 001-14	PHSN 001-14.rbcLa	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN001-14
P. muellerianus	PHSN 002-14	PHSN 002-14.rbcLa	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN002-14
P. urinaria	PHSN 003-14	PHSN 003-14.rbcLa	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN003-14
P. odontadenius	PHSN 006-14	PHSN 006-14.rbcLa	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN006-14
P. niruroides	PHSN 005-14	PHSN 005-14.rbcLa	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN005-14

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Table 7 : Information and links of the *Phyllanthus* species accessions submitted on BOLD Systems for matK gene region

TAXA	PROCESS ID	SEQUENCE ID	LINK/REFERENCE
P. amarus	PHSN 001-14	PHSN 001-14.matK	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN001-14
P. muellerianus	PHSN 002-14	PHSN 002-14.matK	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN002-14
P. urinaria	PHSN 003-14	PHSN 003-14.matK	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN003-14
P. odontadenius	PHSN 006-14	PHSN 006-14.matK	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN006-14
P. niruroides	PHSN 005-14	PHSN 005-14.matK	http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN005-14

IV. Discussion

Barcoding, identification and validation of *Phyllanthus* species using the concatenation of the plastid Ribulose bisphosphate carboxylase large chain (rbcL) and Maturase K (matK) chloroplast genes sequences are seen as a promising tool for the authentication plant species and ensuring better quality herbs and pharmaceuticals. This is otherwise known as DNA fingerprinting apart from identifying alterations in the genotypes of plant species, is also used for the betterment of drug-yield by tissue culturing and of which *Phyllanthus* is certainly one of the most useful herbs in the world. Barcoding results showed that rbcL and matK barcode regions can be used to identify the various species. Barcoding can, with 100% certainty, be used to distinguish between natural growing *Phyllanthus* species in Nigeria. The majority of *Phyllanthus* species may be easily and correctly identified based on morphological traits, but in the vegetative state identification by an inexperienced person become problematic, especially since some of the leaf-characteristics overlap between species. One such example is the nature of the leaf apices, that are characteristic of *P. urinaria* and *P. niruroides*, but that may not only also be absent in both species but also sometimes occur in other species. The leaf apices of both these species are mucronate shaped and as well have reddish, free stipules. These two species, as well as many of the other species, can very easily be misidentified, especially at the seedling stage. Also, it is very important to note that *P. urinaria* have been recorded to be used as fish poison in India, Indo-China, Malaysia and Indonesia (Awomukwu *et al*., 2014; Burkill, 1994) while *P. niruroides* has recorded no toxic effect but medicinal. For this reason it is necessary to identify the appropriate DNA-barcoding regions in *Phyllanthus*, to explore the most appropriate analysis method and to create a proper database for the genus.

From our research study, it was observed that some researchers have published *P. amarus* as *P. niruri* in BoldSystems. Most published sequences were actually mined from GenBank. This might further create confusion in validation and proper nomenclature of the species if not revealed early enough. Also, the results from BLAST on GenBank and BoldSystems showed that *P. amarus* and *P. niruri* may have been ignorantly misquoted or misidentified as separate taxa by different researchers in various parts of the world as regards to its taxonomic relevance. Moreover, from the morphological point of view, *P. amarus* shared similar characteristics with *P. niruri* than any other species in the genus (www.tropilab.com/black-cat.html). It therefore proposed that *P. niruri* should be subsumed to *P. amarus* due to its overall 100% similarity in the DNA sequence data based on the rbcL and matK gene regions. These species are not separate taxa but rather one species of the same genus. An authentic barcode data is highly essential in taxonomic identification and validation. This will help clear confusion encountered in morphological identification of species when plant species are ambiguously identified. The close morphological similarities of members of the genus *Phyllanthus* in Nigeria often times lead to confusion in species identification and collection. Existing taxonomic on the genus in the country is based on morphological characters documented in Hutchinson and Dalziel (1963), which reported some indeterminate species in which *P. amarus* and *P. niruri* were enlisted as separate species according the West African Flora. Reassessment of the members of this genus is therefore worthwhile. We have begun this by subjecting species within the southern region of the country to DNA characterization and barcoding. Other *Phyllanthus* barcodes submitted on BOLD were compared with our accessions and only *P. amarus* and *P. urinaria* had previous records of other authors. *P. muellerianus, P. niruroides* and *P. odontadenius* had no previous submitted sequence records on BOLD and therefore were the first time to be registered and submitted on the website (http://www.boldsystems.org).

DNA of interest can be stored as germplasm, which is then used for future cultivation (Henry, 2001).

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In addition, germplasm can be used for the conservation of selected plant species, which are endangered. Although morphology, anatomy and phytochemistry can be used as useful tools in biosystematics but factors such as soil, climate and adaptability dictate the viability of a particular species and subsequently its contents but such discrepancies cannot be tolerated by DNA barcoding and fingerprinting technique. DNA barcoding has been found to be useful in identifying species, which may have morphological or anatomical difficulties.

It is important to reemphasize the fact that current information is lacking on members of the genus *Phyllanthus* in Nigeria. The efficiency of a two-barcode should be investigated but due to the size of the genus, it is likely that a three or more gene barcode will be necessary. The concatenation of the rbcL and matK chloroplast genes had always been the desirable choice of locus in BoldSystems (Paul *et al*., 2003). Currently in Nigeria, there is need for taxonomists to employ the system of molecular identification of every plant in order to establish a standard genetic library. This will help to curtail the confusion created by ambiguous morphological identification. Moreover, it will also aid to review obsolete literature in the field of taxonomy because recent discoveries will eradicate wrong information on overlapping species identification, nomenclature and classification.

V. Conclusion

The rbcL and matK chloroplast regions are very good barcoding candidate to distinguish the genus *Phyllanthus* in Nigeria because the genus has remained an interesting and challenging one. In addition, the gene markers, rbcL and matK have proved useful in identifying unknown species in the genus. With this type of analysis, identification will never be a problem because it can effectively be used to query sample belonging to any one of the properly delimited species, i.e. *P. muellerianus*, *P. odontadenius, P. niruroides*, *P. urinaria* or *P. amarus* and should be equally effective as an identification tool as the tree-based method. Other genera in the Nigerian Flora is worthy of investigation as only few records exist at the moment. Knowledge of this information will help guide other scientists in the pharmaceutical industries and herbal practitioners who may wish to investigate these plants for further research.

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Global Journal of Science Frontier Research: G
Bio-Tech & Genetics
Volume 15 Issue 1 Version 1.0 Year 2015
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN:2249-4626&Print ISSN:0975-5896

Genomic Diversity and Affinities Among Four Dravidian Tribal Populations of South India: A Study on Eighteen Autosomal DNA Markers

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Abstract- Polymorphic DNA markers are widely used to study genomic diversity, as most of them are selectively neutral, more ubiquitous than polymorphic protein and enzyme markers. Therefore, it is of great interest to study the genomic diversity and their relationships among the four south Indian tribal populations DNA samples from 190 unrelated individuals belonging to four Dravidian tribal populations viz. Malaikuravan, Malasar, Palliyan and Kattunaikkan were analysed by nine human specific insertion / deletion polymorphic loci and nine restriction site polymorphic loci to ascertain their genomic diversity and affinities with other Indian tribal populations. The results indicate that most of the studied loci are highly polymorphic in terms of allele frequencies (average allele frequency=0.51) and average heterozygosity (H_T = 0.49) in all the study populations. The genomic diversity (G_{ST}) of the four tribal populations was comparatively low (0.032).

Keywords: *genomic diversity, dravidian tribes, heterozygosity, genetic distance, alu indels, RSPs.*

GJSFR-G Classification : *FOR Code: 060408*



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Genomic Diversity and Affinities Among Four Dravidian Tribal Populations of South India: A Study on Eighteen Autosomal DNA Markers

A. Krishnaveni [α] & K. Prabhakaran [σ]

Abstract- Polymorphic DNA markers are widely used to study genomic diversity, as most of them are selectively neutral, more ubiquitous than polymorphic protein and enzyme markers. Therefore, it is of great interest to study the genomic diversity and their relationships among the four south Indian tribal populations DNA samples from 190 unrelated individuals belonging to four Dravidian tribal populations viz. Malaikuravan, Malasar, Palliyan and Kattunaikkan were analysed by nine human specific insertion / deletion polymorphic loci and nine restriction site polymorphic loci to ascertain their genomic diversity and affinities with other Indian tribal populations. The results indicate that most of the studied loci are highly polymorphic in terms of allele frequencies (average allele frequency=0.51) and average heterozygosity (H_T = 0.49) in all the study populations. The genomic diversity (G_{ST}) of the four tribal populations was comparatively low (0.032).

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I. Introduction

India represents one of the most diverse regions in the world, wherein populations exhibit enormous diversity in terms of language, culture and ethnicity. The contemporary Indian population is divided into tribal and non-tribal groups. Tribal populations constitute 8.2% (Census of India 2001) of the total population and are considered to be the indigenous populations of India (Fuchs 1964; Thapar 1966; Ray 1973). The total number of tribal groups is estimated to be about 450 (Singh 1992). Population groups inhabiting Tamil Nadu have the distinction of belonging to the Dravidian linguistic family and are predominantly of Australoid ethnicity. The pattern of genetic variation among the tribal population is complex. The contemporary Dravidian speaking tribes of Southern and Central India may be descendants of the original inhabitants of the Indian subcontinent (Fusch 1973).

Human specific insertion/deletion polymorphisms have been used in this study are known to be selectively neutral in nature and the utilization of widely spaced Restriction Site Polymorphism's (RSPs) as "genetic signspots" promised to the successful mapping of human genome in population studies. The recent insertion of mobile elements of Alu family in the human genome provides a distinct class of polymorphisms in the human genome (Deininger et. al. 1999). Alu insertion polymorphisms are ideal markers because retroposition produces infrequent, irreversible and widely distributed insertion elements, each with known ancestral state (Batzer et al. 1994; 1996; Stoneking et. al 1997; Melton et. al 1998; Novict et. al 1996; 1998). Alu element insertions have been linked to several genetic diseases including haemophilia, hypercholesterolemia and various cancers [Deininger and Batzer 1999; Belancio et al. 2008]. While multiple diseases have been attributed to Alu element insertions, their most important role may be in shaping human genome architecture.

Alu elements represent an important source of human genomic variation (Batzer and Deininger 2002) and forensic identification by using insertion-deletion polymorphisms (Murthy et al. 2015). Of increasing significance is the link between these transposon-mediated mutation and disease (Singh et al. 2014). Alu (member of a SINE family) insertion / deletion polymorphisms offer several advantages over other nuclear DNA polymorphisms for human evolutionary studies. They are rapid, simple and stable with newly inserted elements and rarely undergo deletion. Thus, the use of independent DNA polymorphisms provides a body of knowledge in looking back to human history. As they are short enough to show very little or no recombination and behave as blocks that might have ancient origins (Tishkoff et al. 1996a). It is recently proved that ACE deletion / deletion polymorphism could affect the athletic ability in Turkish population (Inanir et al. 2014). Restriction maps of DNA regions will indicate not only the presence or absence of restriction sites, but also the presences of transposable elements and the presence of deletions and insertions. The present study is aimed to investigate the genomic diversity, genetic differentiation and genomic affinities of four south Indian tribal populations based on nine human specific Alu insertion / deletion polymorphisms and nine restrictions site polymorphisms (RSPs) in the nuclear genome.

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II. Material and Methods

a) DNA extraction and PCR amplification

Blood samples (5 -10 ml veniepuncture) were collected from 190 healthy unrelated adult volunteers from four endogamous Dravidian speaking tribal population of south India with prior informed consent. The tribal groups are confined in the villages of hilly tracts and valleys of four different districts of Tamil Nadu, India. They include Malaikuravan (n = 53) from Thiruvannamalai, Malasar (n = 46) from Anaimalai in Coimbatore, Palliyan (n = 50) from Theni and Kattunaikkan (n = 41) from Chennai (Figure 1). Genomic DNA was extracted from whole blood by using the Non - enzymatic salting out procedure method and was suspended in 10mM Tris and 0.1mM EDTA for genotyping. All the eighteen polymorphic loci were genotyped by a standard 30-cycle PCR. Appropriate annealing temperatures, additives were optimized for each system and standardized according to the laboratory condition. PCR amplified segment of all the nine RSPs loci were subjected to digest with 10 units of appropriate restriction enzymes. The PCR protocols have been followed as reported by Stoneking et al.1997 and Majumder et al.1999. Amplified product was then subjected in to agarose gel (containing EtBr stained) electrophoresis at 100V. Gel was visualized under UV light and documented.

The polymorphisms of each major type (Alu InDel and RSPs) are unlinked, and nearly all are located on separate chromosome arms. For the present study nine Alu Insertion- Deletion polymorphisms namely FXIIIB, ACE, TPA25, mtNUC, PV92, APO, PLAT, D1 and CD4; nine RSPs namely PSCR (Taq I), ESR (Pvu II), NAT(Kpn I), HOXB4 (Msp I), T2 (Msp I), CYP1A1 (Msp I), LPL (Pvu II), ALB (Hae III) and ADH2 (Rsa I) were analyzed. The loci and appropriate primers and protocols have been described elsewhere (Jorde et al. 1995; Stoneking et al. 1997; Kidds et al. 1998; Majumder et al. 1999b; Watkins et al. 2001; Vishwanathan et al. 2003).



Figure 1 : Showing sampling location with reference to south India

b) Statistical Analysis

Allele frequencies at each of the nine loci were calculated by direct gene counting method and genotypes were analysed for Hardy-Weinberg Equilibrium (HWE). Nei's expected heterozygosity (Nei 1973) of population was analyzed using Pop Gene version 1.32 software (Yeh et al. 1999). To assess the extent of gene differentiation among the population groups, Nei's measure of gene diversity was calculated separately for each locus and all loci considered jointly. The genomic affinity among the populations was assessed by dendrograms which were constructed by neighbour joining (NJ) tree (1000 replicates) method using DISPAN programme (Ota 1993). Tree reconstruction methods depict population relationships as a series of bifurcations, which are commonly interpreted as population split; however, it is important to realize that clusters of populations in such trees could arise from migration instead of shared ancestry. The Harpending and Ward (1982) model was followed to understand the pattern of gene flow.

III. Results and Discusstion

The allele frequencies of (Alu insertion/deletion loci and RSPs) all the loci are polymorphic in all the populations are given in Table 1. Hardy-Weinberg

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equilibrium was tested using the χ^2 test for goodness of fit after bonferroni correction. It was observed that fifty three values are significant at 5 percent level. NAT locus exhibits maximum variation across the populations ranging from 20% among Kattunaikkan to 57.3% among Pallian. Alu PV92 locus show least variation between populations ranging from 55% among Pallian to 60% among Kattunaikkan. The allele frequency distribution pattern of these populations is quite similar, which are observed with other Indian populations (Majumder et al. 1999; Watkins et al. 2001; Vishwanathan et al. 2003; Veeraju et al. 2008; Saraswathi et al. 2008: Kanthimathi et al. 2008; Yadav and Arora 2011; Dada et al. 2011; Kshatriya et al. 2011; Panjalia et al. 2012).

The heterozygosity at each locus and the average heterozygosity for overall loci for the study population are given in Table 2. The average heterozygosity for each locus was found to vary from 0.2 to 0.5. In most of the cases the heterozygosity for a biallelic marker has attained the maximum attainable value (0.5). However, the average heterozygosity for all the loci in each of the populations ranges from 0.469 among Malasar to 0.481 among Malaikuravan. The average heterozygosity values range from 46.9 % to 48.3 % reflects the genetic heterogeneity.

The amount of genetic differentiation among the populations, G_{ST} values (a measure of the inter-population variability) for each polymorphic locus, along with observed heterozygosity (H_S) with in populations and total genomic diversity (H_T) were calculated and presented in Table 3. The G_{ST} values calculated for each locus determines the degree of gene differentiation between the populations and it varies between 0.39 % at the CD4 locus to 9.4 % NAT locus. When all the loci are considered together, 3.2 % of the total genomic diversity is attributable to the inter-population variations. The total genomic diversity (H_T) among the subpopulation is ranging from 0.458 (CYP1A1) to 0.4999 (ADH2) whereas, H_S value determined between individuals within population ranging from 0.423 (CYP1A1) to 0.496 (Alu mtNUC).

There is significantly greater inter individual variation within each study population than between the populations, hence the extent of population differentiation and the incident of average G_{ST} value for all markers are rather low in the four tribal groups 0.032 (3.2%) or moderate. Earlier studies have reported G_{ST} values ranging from as low as 2.0 % (Kshatriya et al. 2011) among eight western Indian tribal populations, to as high as 8.3 % among five south Indian tribal populations (Vishwanathan et al. 2003).

Table 1 : Allele frequencies and Hardy-Weinberg χ^2 goodness of fit values at eighteen polymorphic loci in four south Indian tribal study populations

Locus	Population											
	Malaikuravan			Malasar			Palliyan			Kattunayakkan		
	n^a	P(+)	χ^2	n^a	P(+)	χ^2	n^a	P(+)	χ^2	n^a	P(+)	χ^2
Alu FXIIIB	80	0.5750	0.270	68	0.5588	5.786*	84	0.5476	11.634*	78	0.3846	12.01*
Alu ACE	94	0.691	4..30	72	0.5694	12.12*	86	0.4884	8.890*	78	0.4615	17.784*
Alu TPA25	88	0.5795	0.83	92	0.4891	4.113	100	0.3800	12.02*	82	0.5976	8.016*
AlumtNUC	90	0.4778	0.420	90	0.5222	0.598	84	0.5357	24.920*	82	0.4268	17.380*
Alu PV92	94	0.6489	5.712*	80	0.5875	4.260	78	0.5513	1.936	80	0.6000	8.984*
Alu APO	92	0.6196	5.514*	72	0.5833	6.416*	88	0.3864	2.786	82	0.5000	5.488*
Alu PLAT	94	0.5426	0.889	84	0.5833	18.60*	82	0.5610	10.450*	82	0.4756	0.2065
Alu D1	90	0.5000	0.610	92	0.7826	3.420	68	0.5147	1.580	60	0.4167	2.970
Alu CD4	88	0.4773	1.752	88	0.5000	6.470*	100	0.4800	4.384	72	0.4167	14.260*
PSCR	96	0.475	7.396*	88	0.409	13.48*	80	0.288	12.83*	78	0.295	7.446*
ESR	94	0.5	16.12*	88	0.409	5.739*	78	0.577	14.93*	76	0.395	4.27
NAT	88	0.409	24.56*	86	0.57	6.725*	82	0.573	5.079*	60	0.2	24.9*
HOXB4	96	0.49	29.38*	88	0.545	3.577	72	0.236	12.56*	56	0.429	13.72*
T2	86	0.558	12.69*	78	0.462	17.78*	72	0.444	10.17*	78	0.423	14.93*
CYP1A1	102	0.569	31.79*	84	0.833	10.17*	78	0.692	15.24*	68	0.485	15.23*
LPL	88	0.682	10.93*	78	0.41	12.41*	84	0.476	16.47*	76	0.566	13.98*
ALB	106	0.557	26.52*	80	0.638	20.79*	98	0.429	34.5*	62	0.468	6.513*
ADH2	100	0.41	20.1*	82	0.524	5.4443*	96	0.5	26.17*	72	0.556	10.17*

** Significant at 5% level*

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Table 2 : Heterozygosity at individual locus and average heterozygosity based on eighteen polymorphic loci in each four south Indian tribal populations

Locus	Malaikuravan	Malasar	Palliyan	Kattunaikkan
Alu FXIIIB	0.4888	0.4931	0.4955	9.4734
Alu ACE	0.4270	0.4904	0.4997	0.4970
Alu TPA25	0.4873	0.4998	0.4712	0.4810
AlumtNUC	0.4990	0.4990	0.4974	0.4893
Alu PV92	0.4556	0.4847	0.4947	0.4800
Alu APO	0.4714	0.4861	0.4742	0.5000
Alu PLAT	0.4964	0.4861	0.4926	0.4988
Alu D1	0.5000	0.3403	0.4996	0.4861
Alu CD4	0.4990	0.5000	0.4992	0.4861
PSCR	0.499	0.483	0.410	0.416
ESR	0.500	0.483	0.488	0.478
NAT	0.483	0.490	0.489	0.320
HOXB4	0.500	0.496	0.361	0.490
T2	0.493	0.497	0.494	0.488
CYP1A1	0.491	0.278	0.426	0.500
LPL	0.434	0.484	0.499	0.491
ALB	0.494	0.462	0.490	0.498
ADH2	0.484	0.499	0.500	0.484
All loci	0.4830	0.4690	0.4760	0,4750

Table 3 : Results of gene diversity analysis for individual loci and for all loci jointly considered in the study populations

Locus	G_{ST}	H_T	H_S
Alu FXIIIB	0.0236	0.4994	0.4876
Alu ACE	0.0323	0.4944	0.4784
Alu TPA25	0.0298	0.4997	0.4848
AlumtNUC	0.0070	0.4998	0.4961
Alu PV92	0.0050	0.4812	0.4787
Alu APO	0.0322	0.4990	0.4829
Alu PLAT	0.0065	0.4966	0.4934
Alu D1	0.0764	0.4942	0.4564
Alu CD4	0.0039	0.4980	0.4960
PSCR	0.0276	0.4650	0.4521
ESR	0.0217	0.4982	0.4873
NAT	0.0945	0.4923	0.4457
HOXB4	0.0556	0.4887	0.4615
T2	0.0107	0.4984	0.4930
CYP1A1	0.0752	0.4580	0.4236
LPL	0.0418	0.4977	0.4769
ALB	0.0262	0.4989	0.4858
ADH2	0.0117	0.4999	0.4940
All loci	0.0321	0.4922	0.4764

The genomic affinities among four study populations are represented as Neighbour Joining tree in Figure 2 using allele frequency data of eighteen loci. This tree is divided into two clusters: Palliyan / Malasar and Malaikuravan / Kattunaikkan. It is seen that the genomic affinities among the study populations attest their socio-cultural affiliation. Pairwise genetic distances between the study populations were calculated from the allele frequencies using the DA distance measure and is presented in Table 4. The maximum genetic distance was observed between Malasar and Kattunaikkan (0.0170) and the least between Malaikuravan and Kattunaikkan (0.0083). The genetic distance analysis indicates that the Malaikuravan and Kattunaikkan are closely related where as the Malasar and Kattunaikkan distantly related.

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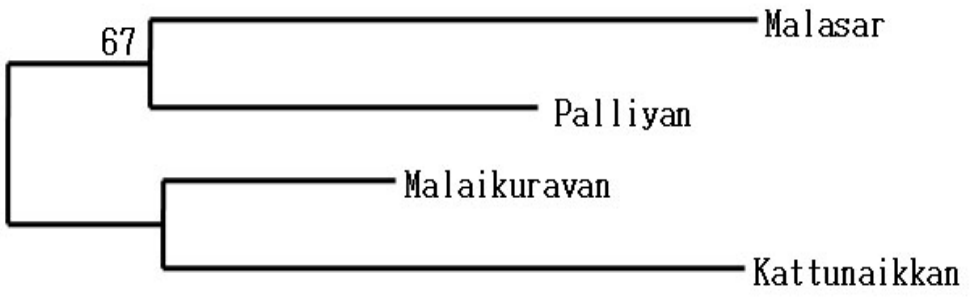


Figure 2 : Neighbour joining tree depicting genomic affinities among four tribal populations of south India

Table 4 : Matrices of pairwise DA distances among four south Indian tribal Populations

	MK	MR	PAL	KN
MK	0.0000			
MR	0.0099	0.0000		
PAL	0.0110	0.0102	0.0000	
KN	0.0083	0.0170	0.0115	0.0000

To determine the genetic relationships of the present study populations with other Indian tribal populations, the data of eleven including six Alu InDel loci (Alu PV92, Alu FXIIIB, Alu APO, Alu ACE, Alu CD4 del and Alu D1) and five RSPs loci (ESR, PSCR, NAT, T2, LPL). The observations that are common with the present study were used to construct the neighbour joining (NJ) tree, reported by Majumder et al. (1999), Mukherjee et al. (2000), Veeraju et al. (2001), Vishwanathan et al. (2003), Saraswathy et al..(2008), Ksatriya et al. (2011) and Dada et al. (2011). The tree consists of the 25 tribal Indian populations including the four study populations is presented in Figure 3. The phylogenetic analysis demonstrates that the study groups genetically stand apart from other Indian tribal group except Kattunaikkan. Most of the tribal populations cluster themselves into their appropriate geographical location of the states. It is generally believed that southern India remained isolated and cushioned from foreign invasions (Rajkumar and Kashyap 2004), although some genetic admixture may have taken place.

Harpending and Ward (1982) derived a regression of heterozygosity, genetic distance. This theory assumes a simple linear relationship between the average heterozygosity of a population from the centroid (the overall mean allele frequency of the populations). If a population is receiving gene from outside (these population used in the analyses) at a higher than average rate, then the heterozygosity will be higher than predicted. A lower than predicted heterozygosity implies that the population is more isolated, there by receiving less gene flow than average.

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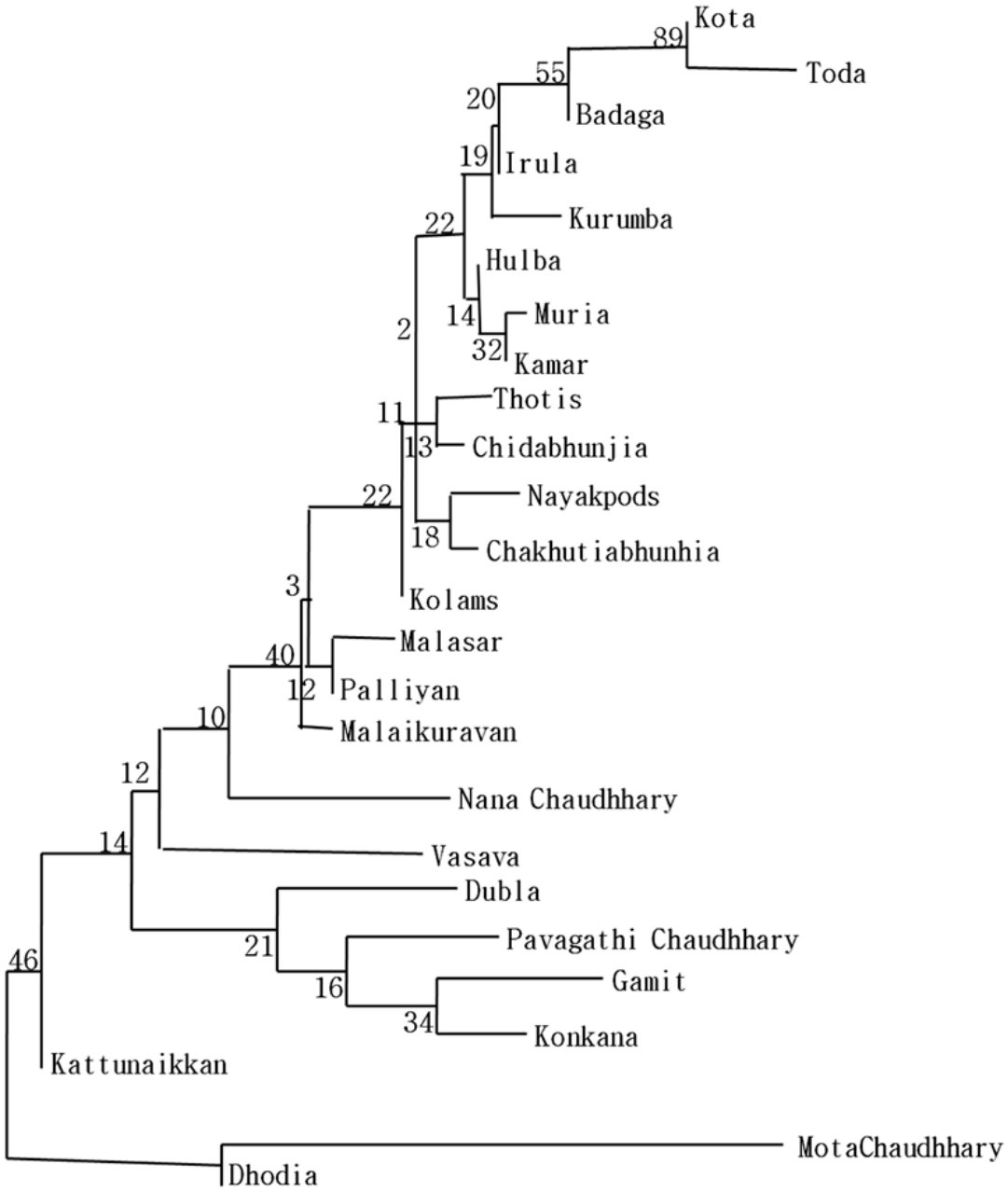


Figure 3 : Neighbour – Joining tree depicting genomic affinities of 25 tribal populations of India based on allele frequency data of six InDel and five RSPs loci

Centroid analysis was also performed by pooling the data of the present study with those of Stone king et al. (1997); Majumder et al. (1999), Mukherjee et al. (2000), Veeraju et al. (2008), Vishwanathan et al. (2003), Saraswathy et al. (2008), Ksatriya et al. (2011) and Dada et al. (2011) based on five alu insertion loci (PV92, FXIIIB, APO, ACE and D1). The genetic distance of the gene frequencies from the centroid and the average heterozygosity (Hs) are presented for each of the 70 populations presented in Figure 4. It is clear from the figure that the average heterozygosities of 11 populations are greater than predicted values. Of these 11 populations, 2 Rajasthan, 2 Western Indian, 3 south Indian, 2 Madhya Pradesh, southern and western Asia each one population. The Indian populations 26 out of 16 (including the study populations) and world populations 34 out of 2 (Stone king et al. 1997) exhibiting average heterozygosity is lower than predicted. The results imply that these four tribal population groups of Tamil Nadu are they completely isolated.

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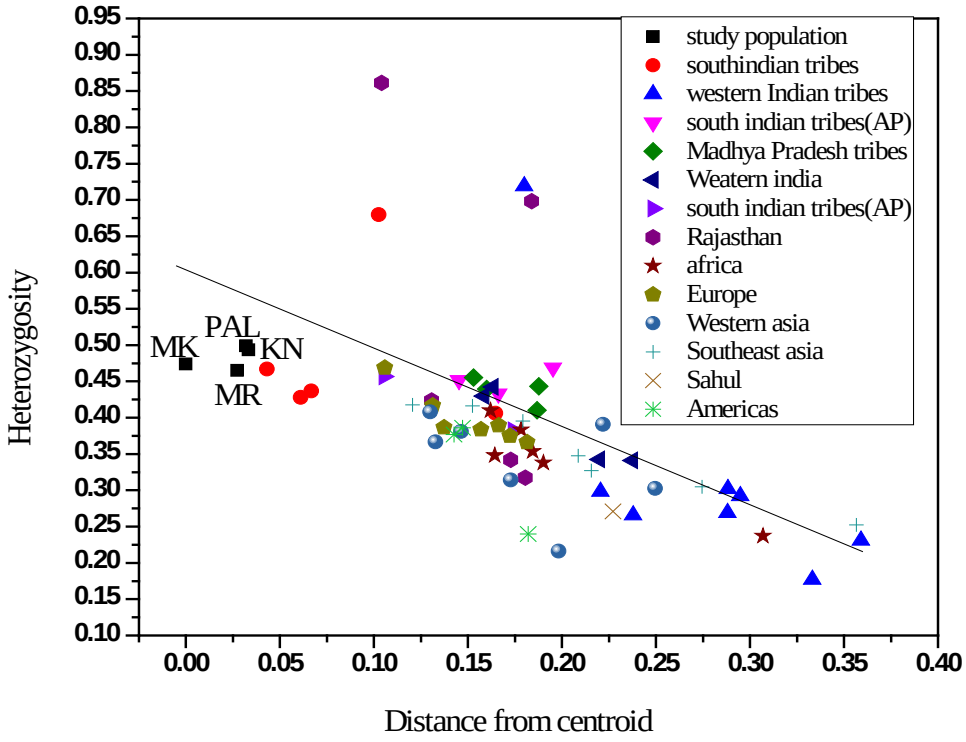


Figure 4 : Genomic affinities of the study population with other Indian tribal and world population based on average heterozygosity Vs distance from centroid at five Insertion loci

The present study reveals that the four Dravidian tribal populations from south India are highly polymorphic, highly heterozygous in nature with lower genomic differentiation. However, similar studies on other population groups of south India with complete representations of populations will help in understanding population relationships, evolutionary and migratory histories of Indian subcontinent.

IV. Acknowledgments

We are grateful to Dr. M.V. Usha Rani, Head, Dept. of Environmental Science, Bharathiar University, Coimbatore, India, Mrs. S. P. Prasanna Vadhani and R. Prabhakaran for their help in collecting the samples and also the participants. Authors also acknowledge the UGC, New Delhi for financial support.

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Global Journal of Science Frontier Research: G
Bio-Tech & Genetics
Volume 15 Issue 1 Version 1.0 Year 2015
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN:2249-4626&Print ISSN:0975-5896

In-Silico Characterization and Homology Modeling of Catechol 1,2 Dioxygenase Involved In Processing of Catechol- an Intermediate of Aromatic Compound Degradation Pathway

By Al-Hakim, Mahmudul Hasan, Rokib Hasan, Md. Hazrat Ali, Muhammad Fazle Rabbee, Marufatuzzahan, Hanif Mohammad Rejwan & Ziaul Faruque Joy

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Abstract- Catechol 1, 2 dioxygenase (EC 1.13.11.1) is an enzyme intended to catalyze the degradation of catechol an intermediate of phenolic compound from *ortho*-mechanisms of the 3-oxoadipate pathway. Catechol 1, 2 dioxygenase plays a key role in the aerobic degradation of aromatic compounds, because it is the substrate for aromatic ring cleavage enzymes and as such it can be the starting point of many peripheral metabolic pathways. So, catechol 1, 2 dioxygenase is deliberated for a solution of environmental pollution occurred by aromatic compounds. In this study, we have focused on the *in-silico* characterization and homology modeling of catechol 1, 2 dioxygenase. The *in silico* analysis was performed by various computational tools and programmes. The physicochemical properties of the selected catechol 1, 2 dioxygenase were analyzed by using ExPASy'sProtParam tool and it was found that the molecular weight (M.Wt) ranges around 35000 Da. Isoelectric Points (pI) exhibits acidic nature and aliphatic index infers that 95% catechol 1, 2 dioxygenaseare stable. The negative value of GRAVY indicates that there will be better interaction with water. Motif analysis of the sequences was conducted by using MEME for predicting probable domain of catechol 1, 2-dioxygenase. Homology modeling of catechol 1, 2 dioxygenase taken from *Pseudomonas aeruginosa* MH38 (AC NO: CDH71767) was performed by I-TASSER. Various bioinformatics programmes and servers like RAMPAGE, PROCHECK and ERRAT were used for analysis and validation of final 3D structures created through homology modeling.

Keywords: *catechol 1, 2 dioxygenase, catechol, aromatic compounds, homology modeling.*

GJSFR-G Classification : *FOR Code: 060102*



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In-Silico Characterization and Homology Modeling of Catechol 1,2 Dioxygenase Involved in Processing of Catechol- an Intermediate of Aromatic Compound Degradation Pathway

Al-Hakim [α], Mahmudul Hasan [σ], Rokib Hasan [ρ], Md. Hazrat Ali [ω], Muhammad Fazle Rabbee[¥], Marufatuzzahan[§], Hanif Mohammad Rejwan [χ] & Ziaul Faruque Joy [ν]

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Keywords: catechol 1, 2 dioxygenase, catechol, aromatic compounds, homology modeling.

I. Introduction

Phenol is one of the most widely used in the organic compounds in existence and is a basic structural unit for a variety of synthetic organic compounds including agricultural chemicals and pesticides. Phenols are aromatic compounds that are characteristic pollutants in waste water and effluents from chemicals, petrochemicals, pharmaceuticals, textiles, and steel industries [3]. The unwholesome and environmentally unacceptable pollution effects of the phenolic effluent have been reported worldwide [4]. Phenol contaminants are relatively soluble in water and accumulate in soil, resulting in extensive surface water, ground water, and soil contamination owing to its severe toxicity [5]. Currently removal of phenol effluents from contaminated sites has been a major environmental issue.

Different techniques have been applied to remove phenolic compounds from polluted areas [6–10]. However, among all, biodegradation process offers the more opportunities to completely destroy the pollutants if possible or at least to transform them to innocuous substance [11], it possess relatively low cost, no chemicals used, and high public acceptance [12]. Phenol and its derivatives are not easily biodegradable because they are toxic to most microorganisms. In higher concentrations, they can even inhibit the growth of microbial strains that are capable of assimilating them. Therefore, phenol is used in practice as an antimicrobial agent. It has been established that phenol can be toxic even in 0.05% concentrations [18]. The metabolism of aromatic compounds, phenol, and its derivatives in particular, is vigorously investigated in prokaryotic microorganisms [17]. A lot of information is accumulated on bacterial species from the *Pseudomonas* genus, which are known for their ability to utilize diverse aromatic compounds as a single carbon source and good degraders of phenol [19–21]. The ability of microorganisms to transform xenobiotics intocompounds that can enter the normal cycle of matter is due to specific microbial enzymes. Thus, the investigation of enzyme reactions including degradation and detoxification of phenol pollutants is the focus of attention for many researchers.

The metabolism of aromatic compounds and its regulation is extensively studied in prokaryotes. In 1973, Stainer reported that the 3-oxoadipate pathway was the main mechanism for degradation of aromatic compounds, including non-substituted phenol [13].

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There are both aerobic and anaerobic microorganisms that are able to complete the phenol degradation process [14]. Phenol-degrading aerobic bacteria have the ability to transform phenol into non-toxic intermediate compounds that enter the Tricarboxylic acid cycle through *ortho-* or *meta*-pathways of degradation [15].

The first step in both pathways is monohydroxylation at the *o*-position of the aromatic ring. The enzyme catalyzing these reactions and having a key role in the aerobic degradation of mono-aromatic compounds is a monooxygenase: phenol hydroxylase (EC 1.14.13.7) [16]. Phenol hydroxylase catalyzes the attachment of a hydroxyl group at the ortho-position of the aromatic ring, thus hydroxylating phenol to catechol. The first intermediate product of phenol degradation is catechol. The dioxygenase enzyme that catalyzes the aromatic ring cleavage of catechol and its derivatives realizes the critical step in the aerobic degradation of aromatic compounds in microorganisms. Two classes of such enzymes are identified on the basis of aromatic ring cleavage mechanisms: intradiol-dioxygenases, (intradiol, i.e., carbon bond between two hydroxyl groups) which use non-haem Fe (III) to cleave the aromatic ring at *ortho-* position regarding the hydroxyl substitutes; and extradiol- dioxygenases, (extra diol, i.e., between one of the hydroxyl groups and a nonhydroxylated carbon) which use non-haem Fe(II) or other two-valent metal ions to cleave the aromatic ring at meta-position with regard to hydroxyl groups.

The enzyme catechol 1,2-dioxygenase described in *Pseudomonas* is highly dependent on ferro- and ferri-ions and has high substrate specificity [22]. Recently, a new catechol 1,2-dioxygenase was isolated form a *Pseudomonas aeruginosa*TKU002 strain capable of assimilating benzoic acid as a single carbon source. The enzyme has unique characteristics, such as very low molecular mass (22 kD), highest activity against pyrogallol, high medium acidity for enzyme production, etc, which distinguishes it from other microbial catechol dioxygenases [23]. Catechol has a key role in the aerobic degradation of aromatic compounds, because it is the substrate for aromatic ring cleavage enzymes and as such it can be the starting point of many peripheral metabolic pathways. Only some of the catechol is subjected to direct aromatic ring scission during the cultivation of microorganisms on phenol as a single carbon source. The rest of the catechol can behydroxylated to pyrogallol, and after that transformed to α- or β-hydroxy-muconates [22,23]. In the previous study we did homology modeling and in silico structural analysis of phenol hydroxylase which is another enzyme strongly involved in aromatic compound degradation system[1].

The study is representing the computational study of catechol 1,2-dioxygenase and its homology modeling. Physiochemical properties, phylogenetic tree construction, motif election and homology modeling and model validation of catechol 1, 2-dioxygenase of *Pseudomonas aeruginosa*MH38(AC NO: CDH71767) are which will help us to better understand of catechol processing in aromatic compound degradation pathway.

II. Methods and Materials

a) Sequence retrieval

The sequences of catechol 1,2-dioxygenase were retrieved from NCBI (National Center for Biotechnology Information). Sequences retrieved was done by BLAST with *Pseudomonas aeruginosa* MH38(Accession numbers: CDH71767.1) in NCBI and 61 sequences are retrieved for further study.

b) Analysis of physicochemical parameters

The different physicochemical properties of catechol 1, 2-dioxygenase enzyme were computed using ExPASy'sProtParam tool and these properties can be deduced from a protein sequence. ProtParam tool is used to analyze various physiochemical properties of industrially important proteins[2]. The ProtParam includes the following computed parameters: Molecular weight (M.Wt), theoretical pI, instability index (II), aliphatic index (AI) and grand average of hydropathicity (GRAVY). The computed isolelectric point (pI) will be useful for developing buffer systems for purification by isoelectric focusing method [26]. The instability index provides an estimate of the stability of our protein. A protein whose instability index is smaller than 40 is predicted as stable; a value above 40 predicts that the protein may be unstable [27]. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). It may be regarded as a positive factor for the increase of thermo stability of globular proteins [28].

c) Motif election

Motif election is very important in case of predicting probable domain ofcatechol 1,2-dioxygenase. Motif election & domain analysis were done using MEME (http://meme-suite.org/) and Pfam (http://pfam.xfam.org/).

d) Construction of Homology Models of Catechol 1,2-dioxygenase

The amino acid sequence of catechol 1,2-dioxygenase of *Pseudomonas aeruginosa* MH38 (Accession numbers: CDH71767.1) was taken for homology modeling and p BLAST was done with PDB (Protein Data Bank). No significant similarity was found and for this why we have done homology modeling by I-TASSER(http://zhanglab.ccmb.med.umich.edu/I-TASSER/).

e) Validation of the models

Homology models generally contain errors in their initial structures. An essential part of homology

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modeling is the verification or validation of the model. Several procedures were used to estimate errors in the 3D models [29]. Their stereo chemical quality and energetic parameters were evaluated to determine whether the bond lengths and angles were within normal ranges, or whether there were many bumps in the models (corresponding to high van der Waals energies). The structures were evaluated and validated by RAMPAGE, PROCHECK and ERRAT [30-32].

f) Rampage

Rampage is a program for visualizing and assessing the Ramachandran plot of a protein structure. It works on the basis of a manually curated set of high quality protein structures and a number of filters, reference phi/psi plots are derived for gly, pro, pre-pro and general residue type and subdivided into 'favoured', 'allowed' and 'outlier' regions will be listed, and a picture of the Ramachandran plotis displayed. The output, high-resolution multi-color Adobe PDF or PostScript file contains the general plot with critical data showing the percentage of residues that occurred in different regions[36].

g) Procheck

PROCHECK aims to assess how normal, or conversely how unusual, the geometry of the residues in a given protein structure, is compared with stereo chemical parameters derived from well-refined, high-resolution structures. The input to PROCHECK is a single file containing the coordinates of the protein structure. The outputs comprise a number of plots, together with a detailed residue-by-residue listing [36].

h) Errat

ERRAT is a program for verifying protein structures determined by crystallography. Error values will be plotted as a function of the position of a sliding 9-residue window. According to the analysis by ERRAT, the final model is significantly improved relative to the initial model. This program examines a PDB file, and generates a score based on the quality of the local structure surrounding each residue, based on the typical ranges of dihedral angles and side chain contacts observed in real proteins, generally speaking, the method is sensitive to smaller errors than 3-D profile analysis but is more forgiving than Procheck [36].

III. Result and Discussion

The physicochemical properties of catechol 1,2-dioxygenase were predicted by using ProtParam tool. The ProtParam includes the following computed parameters: Molecular Weight (M.Wt), theoretical pI, Instability Index (II), Aliphatic Index (AI) and grand average of hydropathicity (GRAVY) (**Table 1**). The physicochemical properties showed that molecular weight of maximum number of catechol 1, 2-dioxygenaseis around 40007.7 Da. The instability index showed that more than 50% catechol 1, 2-dioxygenaseis stable as their instability index stayed below 42. Isoelectric point (pI) is the pH at which the surface of protein is covered with charge but net charge of the protein is zero. The computed pI value showed that catechol 1, 2-dioxygenase are acidic in nature (pH<7). The instability index is used to measure in vivo half-life of a protein [32]. The proteins which have been reported as in vivo half-life of less than 5 h showed instability index greater than 40, whereas those having more than 16 h half-life [33] has an instability index of less than 40. Among the studied catechol 1, 2-dioxygenasemore than 95% protein showed stable nature having more than sixty hours of half-life as the contains instability index less than 40. In case of Aliphatic Index (AI) the studied catechol 1, 2-dioxygenaseshowed the tendency of having a wide range of temperature as showed Aliphatic Index (AI) above 70. GRAVY value of the studied catechol 1, 2-dioxygenaseshowed that maximum) most of them exhibit lower GRAVY value which indicates the better interaction of those proteins with water.

Table 1 : Physicochemical properties of catechol 1, 2-dioxygenase analyzed by Prot Param

Protien name	Organisms	Number of amino acids	Molecular Weight (M.W)	Theoretical pI	Instability index	Aliphatic index	Gravy
Catechol 1,2-dioxygenase	*Pseudomonas aeruginosa MH38*	310	34182.8	5.16	44.09	74.71	-0.594
3'-RNA processing protein	*Pseudomonas*	310	34159.8	5.24	43.95	74.71	-0.577
PA2507, partial	*synthetic construct*	311	34163.8	5.15	44.67	74.47	-0.554
Catechol 1,2-dioxygenase	*Pseudomonas aeruginosa*	309	34128.8	5.24	44.06	74.63	-0.589

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Catechol 1,2-dioxygenase	*Pseudomonas aeruginosa PAO1H2O*	310	34217.0	5.16	46.20	74.06	-0.585
Catechol 1,2-dioxygenase	*Pseudomonas aeruginosa*	310	34189.9	5.24	43.68	75.97	-0.557
Catechol 1,2-dioxygenase	*Pseudomonas aeruginosa WS394*	360	40007.7	8.31	46.90	71.14	-0.687
Catechol 1,2-dioxygenase, partial	*Pseudomonas aeruginosa*	282	31022.3	5.20	36.82	76.21	-0.539
catechol 1,2-dioxygenase, partial	*Pseudomonas aeruginosa*	276	30404.7	5.18	36.95	77.50	-0.536
Catechol 1,2-dioxygenase, partial	*Pseudomonas aeruginosa*	272	29977.2	5.18	37.94	77.57	-0.528
Catechol 1,2-dioxygenase, partial	*Pseudomonas aeruginosa*	205	22005.2	5.10	40.48	76.29	-0.449
Catechol 1,2-dioxygenase, partial	*Pseudomonas aeruginosa*	270	29750.0	5.08	38.14	77.78	-0.522
Catechol 1,2-dioxygenase, partial	*Pseudomonas aeruginosa*	269	29664.8	5.08	38.56	77.32	-0.532
Catechol 1,2-dioxygenase, partial	*Pseudomonas aeruginosa*	272	29970.2	5.08	40.06	76.84	-0.543
Catechol 1,2-dioxygenase	*Pseudomonas denitrificans*	310	34006.8	4.84	40.74	81.26	-0.397
3'-RNA processing protein	*Pseudomonas sp. HYS*	310	34241.1	4.89	39.35	83.13	-0.396
Catechol 1,2-dioxygenase	*Pseudomonas alkylphenolia*	310	34139.1	4.82	38.64	82.81	-0.372
Catechol 1,2-dioxygenase	*Pseudomonas sp. LAIL14HWK12:I7*	311	34140.8	4.94	42.43	77.30	-0.395
Catechol 1,2-dioxygenase	*Pseudomonas sp. URMO17WK12:I8*	311	34243.1	5.07	41.03	79.81	-0.394
3'-RNA processing protein	*Pseudomonas fuscovaginae*	311	34293.1	5.29	40.56	78.23	-0.417

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Catechol 1,2-dioxygenase, proteobacterial	*Pseudomonas stutzeri*	313	34481.2	5.09	39.02	78.91	-0.452
Catechol 1,2-dioxygenase	*Pseudomonas stutzeri*	312	34619.6	5.41	39.16	76.35	-0.537
Catechol 1,2-dioxygenase	*Pseudomonas plecoglossicida*	311	34181.8	4.95	40.85	77.27	-0.422
3'-RNA processing protein	*Pseudomonas putida group*	311	34077.0	5.35	35.54	80.45	-0.362
3'-RNA processing protein	*Pseudomonas stutzeri*	312	34331.1	5.35	47.09	77.28	-0.462
3'-RNA processing protein	*Pseudomonas sp. S9*	310	33856.6	4.81	43.47	78.10	-0.367
Catechol 1,2-dioxygenase	*Pseudomonas sp. HMP271*	312	34645.5	5.37	42.30	75.71	-0.542
3'-RNA processing protein	*Pseudomonas stutzeri*	312	34304.0	5.35	35.18	74.49	-0.526
3'-RNA processing protein	*Pseudomonas alcaligenes*	309	34066.8	4.81	40.24	79.00	-0.420
3'-RNA processing protein	*Pseudomonas putida*	311	34277.0	5.07	39.13	78.87	-0.422
3'-RNA processing protein	*Pseudomonas putida*	311	34279.1	5.21	40.23	78.55	-0.423
Catechol 1,2-dioxygenase	*Pseudomonas alcaligenes*	313	34337.5	5.43	32.99	83.29	-0.389
Catechol 1,2-dioxygenase	*Pseudomonas sp. 20_BN*	312	34391.1	5.03	40.83	79.49	-0.475
Catechol 1,2-dioxygenase	*Pseudomonas putida*	311	34263.0	5.07	40.25	80.13	-0.393
3'-RNA processing protein	*Pseudomonas stutzeri*	312	34577.5	5.21	41.40	80.10	-0.477
Catechol 1,2-dioxygenase	*Pseudomonas xanthomarina*	312	34584.5	5.49	42.06	74.78	-0.526

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3'-RNA processing protein	*Pseudomonas putida group*	311	34320.1	5.14	41.85	79.16	-0.421
Catechol 1,2-dioxygenase	*Pseudomonas taeanensis*	312	34149.0	4.89	49.08	81.09	-0.343
3'-RNA processing protein	*Pseudomonas putida*	311	34264.1	5.14	38.44	79.49	-0.401
3'-RNA processing protein	*Pseudomonas chloritidismutans*	312	34614.6	5.49	43.74	74.78	-0.527
3'-RNA processing protein	*Pseudomonas putida*	311	34387.2	5.13	44.86	78.55	-0.440
3'-RNA processing protein	*Pseudomonas mendocina*	309	34088.1	5.22	41.94	78.35	-0.370
Catechol 1,2-dioxygenase	*Pseudomonas pseudoalcaligenes*	309	34240.2	4.86	34.88	81.81	-0.393
Catechol 1,2-dioxygenase	*Pseudomonas pseudoalcaligenes AD6*	309	34083.1	5.29	38.76	78.96	-0.390
Crystal Structure Of Catechol 1,2-dioxygenase	*Pseudomonas Arvilla C-1*	311	34312.2	5.20	40.18	79.49	-0.390
3'-RNA processing protein	*Pseudomonas putida*	311	34361.1	5.20	43.83	78.55	-0.446
3'-RNA processing protein	*Pseudomonas stutzeri*	312	34333.1	5.15	39.35	78.85	-0.482
Catechol 1,2-dioxygenase	*Pseudomonas cremoricolorata*	311	34140.9	4.90	43.46	78.23	-0.395
Catechol oxygenase	*Pseudomonas putida*	311	34301.2	5.36	39.11	80.74	-0.390
3'-RNA processing protein	*Pseudomonas monteilii*	311	34236.0	5.07	38.30	79.52	-0.391
Catechol 1,2-dioxygenase	*Pseudomonas*	311	34232.9	4.82	42.58	77.94	-0.435
3'-RNA processing protein	*Pseudomonas sp. EGD-AK9*	313	34514.6	5.42	40.83	82.01	-0.422

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Catechol 1,2-dioxygenase	*Pseudomonas resinovorans*	309	33947.8	4.92	32.51	79.94	-0.388
3'-RNA processing protein	*Pseudomonas mendocina*	313	34684.8	5.33	44.03	81.73	-0.499
3'-RNA processing protein	*Pseudomonas sp. M1*	307	33845.7	5.33	37.28	82.41	-0.415
Catechol 1,2-dioxygenase	*Pseudomonas syringae*	309	34354.3	5.07	41.67	84.66	-0.397
Catechol 1,2-dioxygenase	*Pseudomonas sp. MOIL14HWK12:I2*	311	34290.9	4.78	42.64	77.94	-0.445
Catechol 1,2-dioxygenase	*Pseudomonas sp. URMO17WK12:I3*	309	34577.5	5.33	35.34	73.62	-0.537
3'-RNA processing protein	*Pseudomonas thermotolerans*	314	34952.1	5.22	44.83	82.13	-0.439
Catechol 1,2-dioxygenase	*Pseudomonas syringae*	309	34180.0	5.10	42.44	79.90	-0.475
Catechol 1,2-dioxygenase, partial	*Stenotrophomonasmaltophilia*	314	34513.4	5.23	41.63	79.65	-0.429

Motif helps to find out the functional domain of proteins and also motif represents the conserved pattern in protein sequences through which we can design degenerate primer of those protein sequences. Motif analysis of the sequences was conducted by using MEME. This is well known fact that E-value describes the statistical significance of the motif. According to Baker et al [34], by default, MEME looks for up to three motifs, each of which may be present in some or all of the input sequences.

Table 2 : MEME result showing sequence logos with width and respective E value

Motif No	Width	E Value	Sites	Sequence Logo
1	50	1.6e-2998	61	
2	50	1.0e-2920	61	
3	50	7.2e-2496	61	

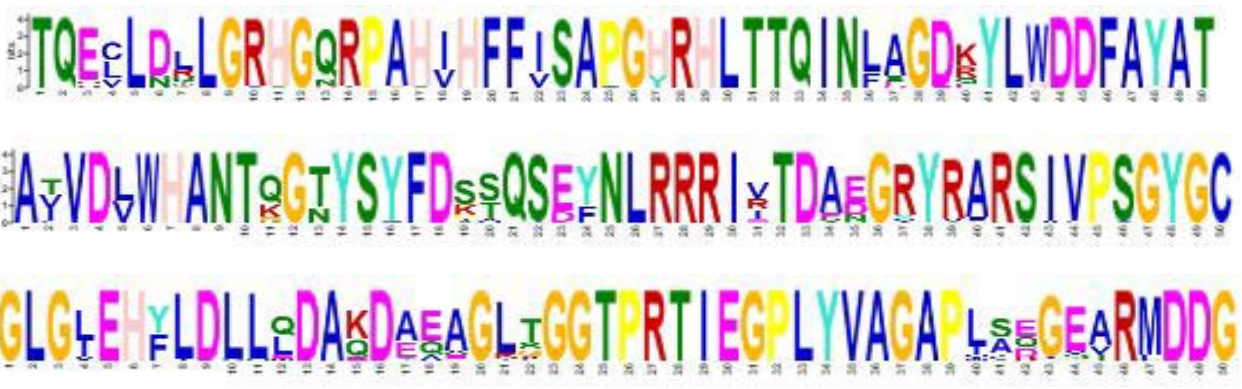


Figure 1 : Three motif of Catechol 1, 2-dioxygenase generated by MEME

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MEME chooses the width and number of occurrences of each motif automatically in order to minimize the 'E-value' of the motif which increases the probability of finding an equally well-conserved pattern in random sequences. In our experiment, motif overview has shown in 'Table 3' describing 1.6e-2998 E-value of motif one, 1.0e-2920 E-value of motif two and 7.2e-2496 E-value of motif three. E-value, width, sites, sequence logo and regular expressions are given in 'Table 2'.

Catechol 1, 2-dioxygenase3D structure is very important in understanding it interactions, functions and their localization. Homology modeling is the most common structure prediction method. *Pseudomonas* species *Pseudomonas aeruginosa* MH38 (Accession numbers: CDH71767.1 was taken as a representative sequence in the experiment and we generated five 3D models (Figure: 2)of it by I-TASSER. Quality and reliability of structure was checked by several structure assessment methods including Rampage, ERRAT and Z-Score by various parameters and Ramachandran plot analysis.

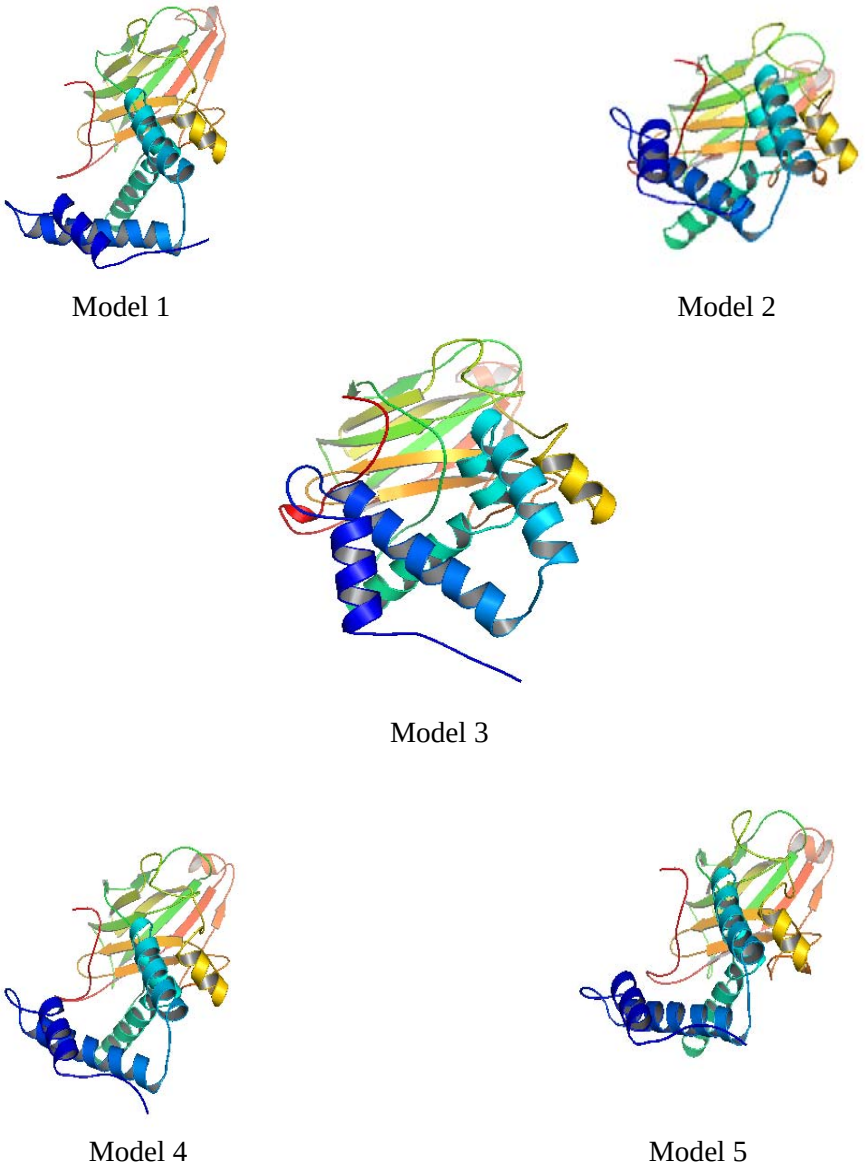


Figure 2 : 3D model of Catechol 1, 2-dioxygenase generated by I-TASSER

Among five models, model 4 has shown better conformational attribute in case of checking by several structure assessment methods. The stereochemical quality of the modeled protein was analyzed by RAMPAGE (Figure 3). Ramachandran plot analysis showed that only 1.0 % residues in outlier region, 2.6 % allowed region and 96.4 % in favored region, indicating that the models were of reliable and good quality.

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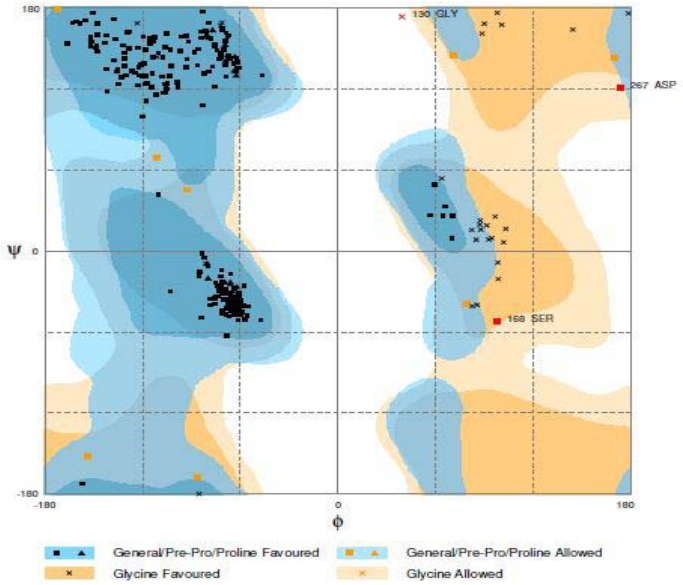


Figure 3 : Favoured amino acid residues in Ramachandran plot for 3-D structure of catechol 1, 2-dioxygenase generated by RAMPAGE

Procheck checks the stereochemical quality of a protein structure by analyzing residue-by-residue geometry and overall structure geometry. This tool was used to determine the Ramachandran plot to assure the quality of the model. The result of the Ramachandran plot showed that 77.7% of residues in favorable region and amino acid percentage in disallowed region as 1.2% (Figure 4).

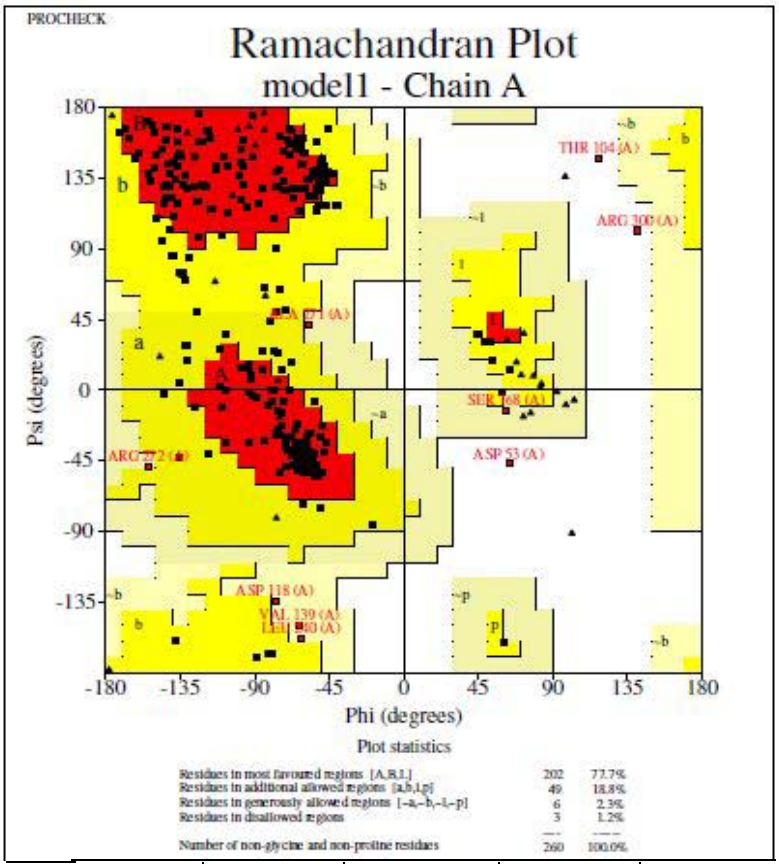


Figure 4 : Showing Ramachandran plot of phi versus psi angles of homology model of catechol 1, 2-dioxygenase generated by using PROCHECK. Color codes are Red color- most favorable regions, yellow color region- allowed region, and pale yellow generously allowed region and white color- disallowed regions

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ERRAT is a protein structure verification algorithm that analyzes statistics of non-bonded interactions between different atom types based on characteristic atomic interaction [35]. ERRAT is a so-called "overall quality factor" for non-bonded atomic interactions, with higher scores indicating higher quality. The generally accepted range is >50 for a high quality model. For the current 3D model, the overall quality factor predicted by the ERRAT server was 86.093 (Figure-5).

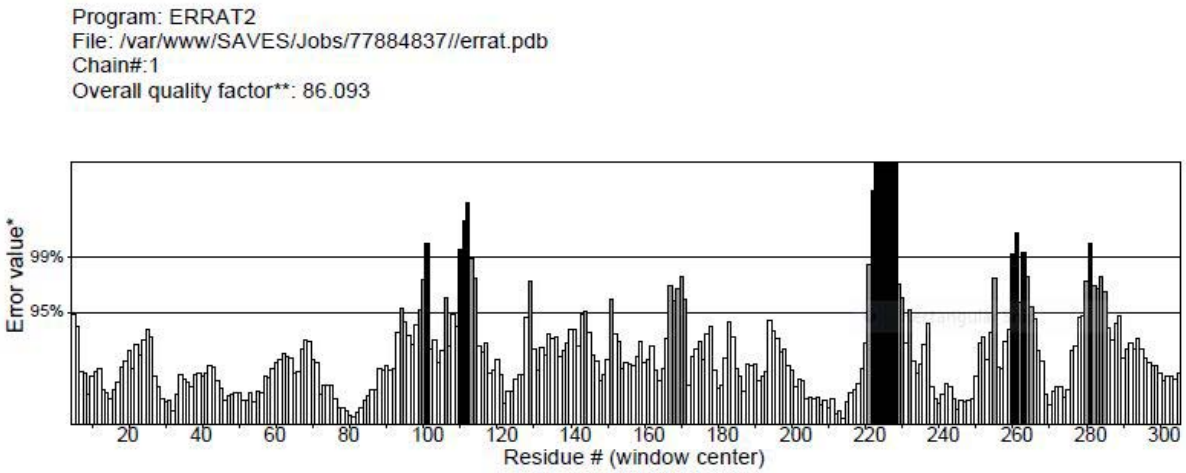


Figure 5 : ERRAT result showing amino acid distribution in error or non-error region for catechol 1, 2-dioxygenase. Black bars identify the misfolded region located distantly from the active site, gray bars demonstrate the error region between 95% and 99%, and white bars indicate the region with a lower error rate for protein folding

IV. Conclusion

In this study, catechol 1, 2-dioxygenase were selected and characterized from physicochemical perspectives.For this enzyme, molecular weight, theoretical isoelectric point, aliphatic index, instability index, Grand Average Hydropathy (GRAVY) was computed and that are essential and vital in providing data about catechol 1, 2-dioxygenase and their properties. Conserved sequences in motifs help us to culminate a significant insight of functional domain and also it may be utilized for designing specific degenerate primers for identification and isolation. Homology modeling generated five structural models and validation technique extols the best one. This 3-D structure may provide a new insight to better understand of structure and function. The study also focused on the future prospects of research in environmental safety concern.

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Global Journal of Science Frontier Research: G
Bio-Tech & Genetics
Volume 15 Issue 1 Version 1.0 Year 2015
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN:2249-4626&Print ISSN:0975-5896

Role of Biomaterials in Neural Stem Cell Fate

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Abstract- In this article we review the types of natural and synthetic materials that are being used in brain tissue engineering applications for traumatic brain injury and stroke. We analyse modifications of scaffolds including immobilizing drugs, growth factors and extracellular matrix molecules to improve central nervous system regeneration and functional recovery. This review attempts to outline the varieties of biomaterial parameters that are applied as biophysical and biochemical signals to direct NSC fate and behaviour. The understanding on the interaction of NSCs decision and biomaterial parameters is helping to advance NSCs-based clinical approaches for nerve tissue regeneration and repair.

Keywords: biomaterial scaffold, central nervous system, neural stem cell fate, traumatic brain injury, neural tissue engineering, hydrogels, cell transplantation, cell survival.

GJSFR-G Classification : FOR Code: 090301



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Role of Biomaterials in Neural Stem Cell Fate

Athira K. S. [α] & Paulose C. S. [σ]

Abstract- In this article we review the types of natural and synthetic materials that are being used in brain tissue engineering applications for traumatic brain injury and stroke. We analyse modifications of scaffolds including immobilizing drugs, growth factors and extracellular matrix molecules to improve central nervous system regeneration and functional recovery. This review attempts to outline the varieties of biomaterial parameters that are applied as biophysical and biochemical signals to direct NSC fate and behaviour. The understanding on the interaction of NSCs decision and biomaterial parameters is helping to advance NSCs-based clinical approaches for nerve tissue regeneration and repair.

We conclude with a discussion of some of the challenges that remain to be solved towards repairing and regenerating the brain. This review seeks to describe the current types of scaffolds and evaluate their use in combination with stem cells for TE applications. Finally, conclusions about the current state of biomaterial scaffolds containing stem cells for TE applications are drawn and suggestions for the future direction of the field are given. An overview of the available biomaterials for use in combination with directed stem cell differentiation as means of replacing diseased or damaged tissues are given. In this review, current and emergent approaches based on stem cells in the field of TE are presented for specific applications of human tissues and organs. The combination of stem cells and TE opens new perspectives in tissue regeneration for stem cell therapy because of the potential to control stem cell behaviour with the physical and chemical characteristics of the engineered scaffold environment. Niche includes a biomaterial with appropriate biochemical and mechanical factors for the cells and tissues studied. In this review, we examine the mechanisms that contribute to the death of transplanted cells. We review both the in vitro data, where biomaterial scaffolds are designed to enhance cell survival, and the in vivo data, where scaffolds are shown to improve cell survival following transplantation into the damaged brain and spinal cord.

Keywords: biomaterial scaffold, central nervous system, neural stem cell fate, traumatic brain injury, neural tissue engineering, hydrogels, cell transplantation, cell survival.

I. Introduction

Brain neurological disorders, such as stroke/cerebral ischemia, traumatic brain injury (TBI) and neurodegenerative diseases, are lack of effective treatments in the past years due to the extensive loss of cerebral parenchyma [1]. Recently, trans-plantation of stem cells has been becoming an important approach for injured brain tissue regeneration. Neural stem cells (NSCs), which have been isolated from various regions in the developing and adult nervous system, are capable to differentiate into all kinds of neural cell types including neurons, astrocytes, and oligodendrocytes, offering promising prospects for the treatment of brain diseases [2]. However, stem-cell therapy for central nervous system (CNS) diseases is of challenge because the blood–brain barrier limits the diffusion of neurotrophic molecules into the brain by traditional oral or intravenous routes. Moreover, the lesion brain cannot afford a suitable microenvironment for NSCs regeneration because inflammation, glial scar formation, release of inhibitory molecules, and absence of growth-guiding astrocytes [3]. Therefore, it is necessary to develop advanced biomaterials generating bioactive artificial microenvironments, which closely mimic the natural niche to support NSCs growth without losing ''stemness'' or undesired differentiations.

In the body, stem-cell populations physically reside within instructive local tissue niches that maintain and regulate stem cells fate [4]. Artificial microenvironment of NSCs can be designed with new material syntheses and processing techniques to feature an intense signal to maintain NSCs stem cell fate, or a myriad of signals that address the biologically relevant sequence of events leading to stem cell lineage commitment. Biomaterials could also be used as delivery vehicles for NSCs transplantation to deliver trophic factors, support residual neurons around the injury site, maintain of replacement cells, provide contact guidance for directed axonal outgrowth, and minimize hostile inflammatory reactions. In this article we review progress to date employing tissue engineering to promote cell replacement using neural precursors (NPs) to restore neurological function after traumatic brain injuries and stroke. The therapeutic value in transplanting NPs is extremely high due to the inability of neurons to undergo mitosis and the incapacity of the brain to repair large injuries on its own.

The study of NSC-biomaterial interactions would advance our understanding on the mechanisms of NSCs-fate specification and self-renewal which could in turn pave way for the rational design of new scaffolds that encourage successful incorporation, survival, and integration of NSCs into diseased or injured regions of the CNS [5]. Specifically, changes to one or more parameters at the initial time point of cell culture could ultimately influence long-term functional differentiation and gene expression [6]. This paper reviewed the recent progresses in the studies of biomaterials as NSCs

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artificial niches to direct NSCs fate for brain tissue engineering (TE). We first summarized the components of the natural NSCs niche, as it is necessary to establish a solid conceptual framework for artificial niches design. We then outlined various biomaterial parameters both biophysically and biochemically directing NSCs fate in vitro, as well as functionalized scaffolds facilitating the in vivo transplantation of NSCs for brain TE. The adhesive properties of catecholamine, representatively as poly (dopamine), have helped to realize the efficient immobilization of biomolecules onto surfaces with various chemistries [7]. Importantly, the facile approach of using a catecholamine group as a coating agent not only allows flexibility in the selection of the substrate materials but is also an inexpensive and eco-friendly process [8]. The spatial arrangement of the surface adhesiveness may result in the patterned regulation of cellular behaviours, including differentiation, proliferation, and migration [9].

The discovery of NSCs, which have the ability to self-renew and differentiate into all types of neural lineages, offers promising prospect for the treatment of brain neurological disorders such as stroke/cerebral ischemia, TBI and neurodegenerative disorders. However, only limited number of NSCs could survive or propagate due to tissue inflammation or blood–brain barrier. Therefore, it is necessary to develop an appropriate culture system that highly mimics the natural NSCs niche to direct stem cell fate and behaviour for nerve regeneration. Both biophysical and biochemical properties of the NSC niche, including topology, mechanical properties, bioactive molecules, and their spatial and temporal presentations should be considered for the design of functionalized scaffolds, which could not only serve as the delivery vehicles of NSCs but also stimulate specific cellular responses at the molecular level, such as support endogenous or exogenous cells proliferation, migration and homing, even promote the growth of axon at the injured brain site.

II. Brain Injuries

Approximately 26 million people sustain traumatic brain injuries each year as a result of falls, motor vehicle accidents, being struck by objects or assaults. An additional 1.2 million individuals are affected by stroke, of which 80% are ischemic and are of varying severity. These numbers do not include major brain injuries caused by infections, tumours or other CNS diseases that account for another large population. Brain injuries are generally classified as mild, moderate or severe depending on damage sustained. Majority of TBIs are mild, resulting in a change in mental status or state of consciousness. Severe brain injuries may cause amnesia, long periods of unconsciousness, irreversible changes in cognitive (attention and memory), motor (coordination, balance, and limb weakness/paralysis) and sensorimotor function (vision, hearing, and touch), alteration in emotions (anxiety, depression and personality changes) and even death [10].

a) Pathophysiology

Individuals who do not die within the first few months after sustaining a severe brain injury are often left with disabilities and a poor prognosis for the duration of their lives. The acute affects can be observed within the first hours after injury and can be amplified within the first several weeks, generally attributed to the pro-inflammatory response to the injury that can last for months or years [11]. Neuronal damage and cell loss have been extensively documented and characterized in the cerebral cortex, the hippocampus and the thalamus in the acute phase following experimental brain injury [12]. The primary damage created by mechanical forces at the moment of the impact is irreversible. In response, immune cells are recruited to the damaged site, whereupon they release cytokines and chemokines triggering a neuroinflammatory reaction that produces a wave of secondary cell death. After a delay, the astrocytes surrounding the injury begin to produce a glial scar. Once formed, this scar tissue creates an inhibitory environment eliminating the possibilities of axonal regeneration due to the formation of a complex extracellular matrix (ECM) [13]. This prolonged and progressive pathologic cascade becomes the basis for the deficits in cognitive and motor function that begin in the first hours after TBI and may continue for years. Kuruvilla et al.[14] reported that serotonin and gamma amino butyric acid along with autologous bone marrow cells to 6-hydroxydopamine infused rats renders protection against oxidative stress mediated neuronal damage as in Parkinson's disease which makes them clinically significant for stem cell-based therapy. The alterations in dopamine D 1 receptor-binding parameters and gene expression during Parkinson's model were reversed by serotonin and gamma amino butyric acid supplementation [15]. Paul et al. [16] showed serotonin and norepinephrine functionally reversed Dopamine receptors significantly in rotenone induced Hemi-Parkinson's rat.

b) Treatment

After a person sustains an injury, the medical team will provide resuscitation procedures, and stabilize vital functions to minimize secondary damage to the brain. Mechanical ventilation is used to support respiration and to maintain lower intracranial pressure. Sensory devices may be surgically placed into the brain cavity to monitor or control intracranial pressure. Surgery may be required to repair haemorrhaged arteries or to eliminate blood clots. Blood, fluid and bone particles can be removed while damaged tissue, blood vessels or the skull can be surgically remodelled in severe cases

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where there is extensive swelling. Patients are also kept sedated with medications to prevent them from causing any additional injury and to prevent seizures and spasticity. Doctors try to maximize cerebral perfusion pressure and blood flow (which includes oxygen and nutrients being supplied to the brain) while minimizing the swelling caused by pressure that may damage more cells [17]. Pharmaceutical agents also may be used to limit secondary damage to the brain which include: diuretics to reduce edema thus decreasing pressure; anti-seizure drugs to avoid additional brain damage; and coma-inducing drugs because a comatosed brain requires less oxygen to function [18]. Other medications such as analgesics, anti-anxiety agents, anti-depressants, anti-psychotics, muscle relaxants, sedatives and stimulants are also commonly utilized in patients sustaining TBI [19]. To date, there are no therapies capable of replacing the neurons lost to brain injuries, thus making full functional recovery after severe TBI impossible.

III. Tissue Engineering

Chronic limitations of traditional transplantation surgeries still exist due to the lack of appropriate donor tissues, risk of disease transmission, and potential for immune rejection. Tissue engineering, the multidisciplinary application of biology, chemistry, physics, engineering, and medical science, offers an alternative method to overcome these issues [20]. For therapeutic application of TE, engineered tissue is grown either within a patient or outside the patient and subsequently transplanted into the patient. Figure 1 provides a schematic representation of the process of neural tissue regeneration by engineering biomaterial scaffolds. Human cells are harvested from a patient and after in vitro cell culture, cells are seeded onto scaffolds with medium containing chemical stimuli, such as growth factors and differentiation-inducing factors. Scaffolds are three-dimensional (3D) matrices that support cellular growth processes, such as cell adhesion, migration, proliferation, and differentiation, by which cells are colonized onto the scaffold. The cell-colonized scaffold is then implanted into the patient, to regenerate bio-compatible, immunocompatible, and bio functional tissues or organs inside the patient body. Cells and scaffolds are essential to regenerate new tissues with TE. Cells become the primary component of engineered tissue and the scaffold provides cells with an appropriate physical and chemical environment where they can attach to the surface of the scaffold, migrate through the scaffolds' pores, and then proliferate. In some instances, such as stem cell therapy, collaboration of cells and scaffolds with differentiation-inducing factors is essential for stem cells to differentiate into engineered cell lineages and to develop new tissues.

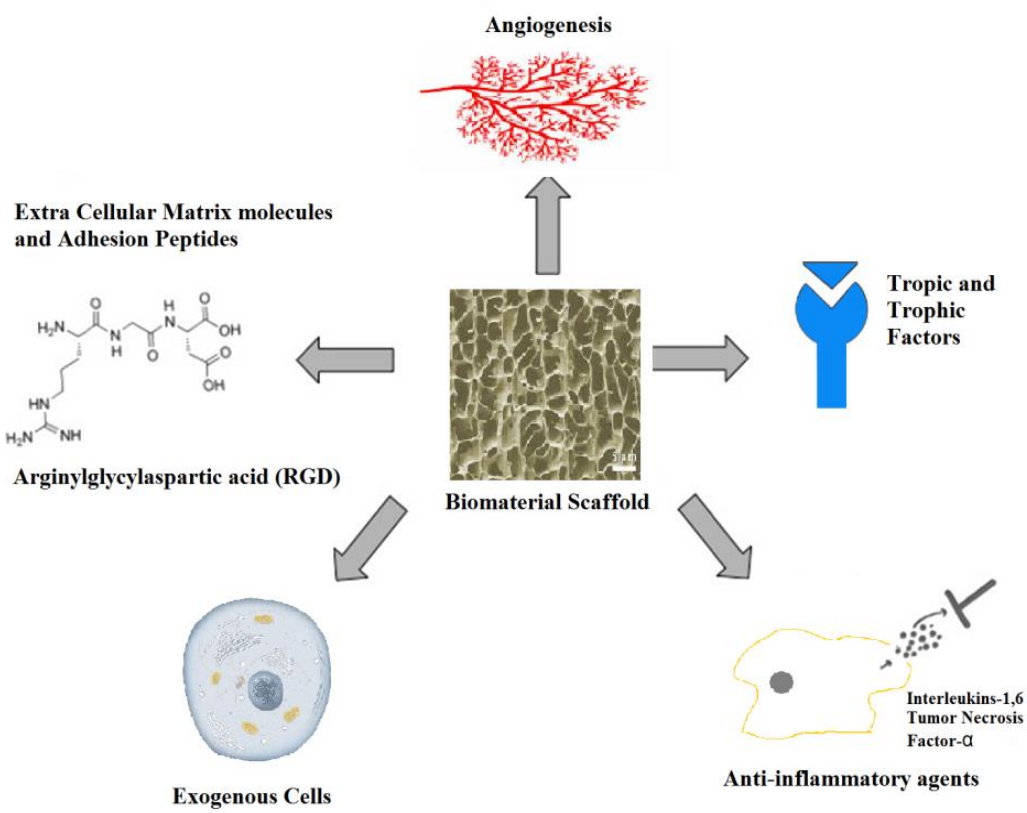


Figure 1 : Engineering Biomaterial Scaffold to Improve Neural Tissue Regeneration

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a) Stem Cells in Tissue Engineering

Although it is difficult to grow some cell types such as cardiomyoctes and hepatocytes in large quantities, stem cells are undifferentiated biological cells that can produce more stem cells (self-renewal) and can differentiate into specialized cells (cell potency). Transforming growth factors beta 1 and 3 (TGF-β1 and TGF- β3), for example, have been reported to enhance the differentiation of Mesenchymal Stem Cells (MSC) to chondrocytes [21] and the chemical agent β-mercaptoethanol (BME) has been used for neural trans differentiation of MSCs [22]. TE may be used for tissue regeneration such as bone, cartilage and neural tissues using degradable biomaterial scaffolds. For example, tubular collagen nerve guides (Neuragen from Integra Life Sciences) were used clinically to treat peripheral nerve injuries and the critical gap length treated by nerve guides was longer than 10 mm in primates and could be further increased by adding fibers or hydrogel with cells [23].

b) Stem Cells in Neural Tissue Engineering

The CNS, consisting of the spinal cord and the brain, is a very unique tissue network with an unusual ECM structure and characteristic soft physical properties which is susceptible to damage, illnesses, and injuries, including traumatic brain injury, spinal cord injury, stroke, Parkinson's disease, and multiple sclerosis [24]. The mechanical properties, structure, and composition of the ECM are effectors of cell function, thus, soft hydrogel scaffolds are utilized for CNS applications to mimic the biochemical and mechanical properties of the CNS [24]. For instance, hydrogel scaffolds made of acrylamide and PEG with arginine-glycine-aspartic acid (RGD) can regulate cell behaviours, such as adhesion, cell renewal, and differentiation of NSCs [25]. Platelet-derived growth factor (PDGF)-AA immobilized agarose scaffolds have been reported to support differentiation of NSC and neural progenitor cells (NPCs) to oligodendrocytes [26]. Sakata et al. [27], preconditioned NPs with interlukin-6 (IL-6) before transplanting those 6–7 hours after transient middle cerebral artery occlusion. The preconditioned NPs were protected from death and they released Vascular endothelial growth factor (VEGF) resulting in increased angiogenesis within the target site. Hydrogel scaffolds made of RADA 16-1 IKVAV (isoleucine-lysine-valine-alanine-valine) have been shown to serve as a guiding cue to direct NSC adhesion and neural differentiation with in vitro and in vivo to direct stem cell differentiation toward neural lineages and to promote the signal transmission among neurons because of electrical conductivity. The hydrogel in a rat brain surgery model enhanced survival of NSCs, reduced the formation of glial astrocytes, and improved brain tissue regeneration after 6 weeks post-transplantation [28]. Electrical stimulation was shown to enhance the proliferation and differentiation of NSCs on thin film scaffolds made of laminin (LN) and single-wall carbon nanotubes (SWCNT) [29]. Bioelectricity has shown to affect intercellular signalling of the nervous system and extended neurite outgrowth compared to cells grown on non-stimulated scaffolds [30].

i. *Adult Neural Stem-Cell Niche*

NSC was discovered in adult nervous system, which broke the curse of brain as a quiescent organ that nothing may be regenerated. Therefore, NSCs for stem cell based therapies in the regeneration of adult brain have drawn much attention recently. Successful application of NSCs therapies clinically would require precise control over the cellular behaviour. The microenvironment of NSCs termed as NSCs niche was therefore extensively studied. In vivo, the sub ventricular zone (SVZ) of the forebrain and the sub granular zone (SGZ) of the hippocampus as two main resources of NSCs act as in vivo NSCs niche, which physically localizes NSCs and maintains their stem-cell fate. Niche could support following functions of NSCs: it maintains NSCs in a quiescent and undifferentiated state to avoid being depleted by aging; niche provide a neurogenic environment for NSCs because large amount of NSCs transplanted into the brain outside the niche are very prone to differentiate into glial cells; and niche is structured so that both the number and type of differentiated progeny can be modulated in response to a diverse array of physiological cues [31].

ii. *Cells in NSCs niche*

In the adult mammalian brain, the SVZ is composed of different types of cells, including a monolayer of ependymal cells lining the ventricle, NSCs, transit-amplifying cells, neural progenitors (neuroblasts), and astrocytes (Figure 2) [32]. All types of cells are not isolated from each other, but mutually connected. The NSCs are relatively quiescent cells that express markers reminiscent of embryonic radial precursors, as well as the glial fibrillary acidic protein. The NSCs give rise to transit amplifying cells, which in turn generate neuroblasts. The neuroblasts migrate in glial tubes to olfactory bulb and generate neurons that integrate into neural circuitry. Researches indicated that a rich plexus of blood vessels snake along and within neuroblast chains in the SVZ. Some of the NSCs and transit-amplifying cells are closely associated with blood vessel that they may receive important signals from the vasculature [33].

iii. *Extracellular matrix in NSCs niche*

The ECM is the most important non-cell component of NSC niche, which could provide anchorage for NSCs adhesion and manipulate the concentration and presentation of signalling molecules to regulate NSCs behaviours. Generally, the ECM is structurally composed of two major components, interstitial matrix and basement membrane, which

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contains adhesive glycoproteins, glycosaminoglycan's (GAGs), and ions. The interstitial matrix of brain ECM is composed of a ternary network of the glycosaminoglycan hyaluronic acid, proteoglycans of the lectican family (brevican, aggrecan, neurocan, versican), and intermingled link proteins of tenascins connecting to cell surfaces. Hyaluronic acid (HA), which works as a ''backbone'' in brain, would bind with tenascins and proteoglycans forming an organized HA-proteoglycan network around the embedded cells [34]. LNs as the main component of the basement membrane in NSCs niche are a family of heterotrimeric proteins that contain one a, one b, and one g chain subunits, found in five a, three b, and three g genetic variants, respectively [35]. LNs not only play an important role as the framework but also have different functions in the signal transportation. For example, finger like processes of basal lamina called fractones extend from blood vessels to contact each stem cell in the niche. Consequently, each stem cell receives at least three different sources of LN signals: from interstitial LNs, from their processes attached to blood vessels, and from the contacting fractones. Other ECM molecules such as the glycoprotein tenascin C, chondroitin sulphate proteoglycans (CSPG), and heparin sulphate proteoglycans also play important roles in the migration, differentiation and proliferation of the NSCs.

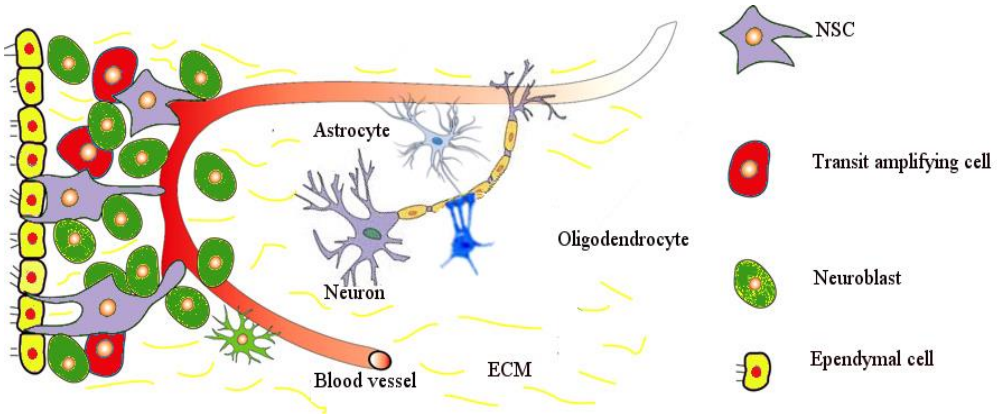


Figure 2 : NSC Niche in Sub Ventricular Zone of Adult Mammalian NSC

iv. *Endogenous stem cells*

Scientists have tried to expand the endogenous stem cells found inside the brain to repair damage after CNS injury. Despite significant work, several problems still exist with this approach. First, few neurons are generated in response to injury, as the vast majority of the new cells that are produced become glia. While infants have significantly larger numbers of NSCs than adults, and thus greater potential for repair [36], the NSCs of the immature brain simply do not produce many new neurons after TBI. Another barrier to regeneration from the endogenous stem cells of the brain is that the pools of NSCs are depleted with age [37]. Arvidsson et al. [38] researched the mechanisms of neuronal repair after stroke in an adult rat model and reported that less than 1% of the destroyed neurons are replaced from the endogenous NPs of the SVZ. Similar results were obtained in rat models of stroke in the immature animal where cell counts of immature neurons vs. mature neurons revealed that greater than 75% of the newly produced neurons failed to survive. Moreover, of those neurons that did survive were predominantly GABAergic inter neurons [39]. In a mouse model of TBI, same pattern was seen [40] and found that SVZ cells proximal to injured area produced a very small percentage of new neurons, while the majority became astrocytes. Whether the newly generated neurons died at injury site or failed to migrate from the SVZ to damaged region is unclear. In the adult brain, neural progenitors have a difficult time migrating to injured cortex due to dense white matter tracts [41].

c) *Brain Tissue Engineering*

A focal TBI results in a large number of dead cells and debris that are localized near the region of impact. Macrophages clear away remnants of dead or dying cells, but the injury creates a harsh, non-permissive environment that lacks nutrients, survival factors and most importantly, a habitable substrate and ECM that they once resided within [42]. This ECM is a scaffold that provides cells with structural and functional support. It is comprised of interconnected proteins and proteoglycans that create a framework that cells adhere to. Attachment to individual components of this matrix transduces mechanical signals that regulate both basic and complex cellular processes. The proteins and proteoglycans that comprise the ECM bind to a number of surface receptors found on cells that can affect proliferation, migration, differentiation, survival and other functions [43; 44]. Although reactive astrocytes produce ECM molecules in the process of generating the glial scar, this ECM is distinctly different from normal ECM in the brain functionally, chemically and mechanically [45].

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IV. Biomaterial Scaffold Structures

Such biomaterial scaffolds can be used to promote the viability and differentiation of stem cells seeded inside-based on both the intrinsic properties of the material and the incorporation of specific cues into the material. NSCs have been isolated from various species such as mice, rats, and humans and from numerous regions in the developing and adult nervous systems including the SVZ, the SGZ of the hippocampus, the cortical neuroepithelium, and the spinal cord [2]. In vivo, the NSC is encompassed by a microenvironment or niche that presents it with a repertoire of diffusible factors, [46] cell-cell interactions, [47] and ECM ligands that bind to cellular receptors and thereby modulate signalling and gene expression [48]. These soluble and solid-phase components of niche collectively regulate cell behaviour and functions including mitosis, apoptosis, migration, and differentiation [49]. NSCs have therapeutic potential to treat disorders and injuries such as Huntington's disease, multiple sclerosis, Parkinson's disease, stroke, and diseases and injuries of the spinal cord [50]. In cell transplantation therapies, NSCs have survived in various regions of the CNS, including the striatum, hippocampus, ventricles, SVZ, olfactory bulb, and cerebellum, [51] and have shown promising results when implanted at the injured/diseased sites in animal models for numerous diseases and injury, such as Sly disease, myelin degeneration, Parkinson's disease, and spinal cord injury [52].

It is becoming increasingly clear that not only the biochemical but also the mechanical properties of microenvironment can modulate cytoskeleton, the adhesion and growth of cells, and even differentiation of stem cells [53]; therefore, it would be desirable to be able to finely tune mechanical properties of culture system. The biochemical and mechanical signals of proteins or materials mimicking the solid phase of native stem cell microenvironment will play a major role in controlling first expansion and then differentiation of stem cells for clinical applications. A scaffold is a 3D matrix that provides the framework and initial structural support for cells to attach, proliferate, and differentiate, facilitating the formation of an ECM [54]. For cell based TE, cells are usually seeded onto scaffolds which are made of materials such as acellular tissue matrices, naturally derived materials (natural biomaterials), and synthetic polymers (synthetic biomaterials). Synthetic biomaterials have tunable mechanical properties, however, the biocompatibility of natural biomaterials is better than synthetic materials, thus, hybrids of natural and synthetic materials are also used for scaffold fabrication. To support tissue regeneration for in vitro stem cell study, differentiation-inducing factors can be loaded into scaffolds to promote and to induce differentiation of stem cells, but these factors under specific circumstances remain indispensable. Achieving success in TE is attributed only to stem cells and scaffolds, suggesting that the effects of differentiation factors may be substituted with suitable scaffold structures [20].

a) Criteria to be used as scaffold

Scaffolds are 3D artificial structures that are created to recapitulate in vivo milieu providing cells with an appropriate microenvironment. Since brain injuries vary in shape and size, scaffolds that form after injection into wound cavity allow for a one size-fits-all solution. In an ideal biomaterial design, a list of desirable functions of a scaffold for a particular biological application should include nontoxicity, biocompatible with transplantable cells and brain tissue environment, maintain stemness of transplanted cells, controlled biodegradation, injectable, porous and remain local.

b) Natural Biomaterials

Tate and Shear, were some of the first investigators to use stem cells for brain TE in models of TBI. They produced collagen gels that contained either fibronectin and/or LN and showed that these scaffolds increased the survival of transplanted mouse NPs compared to NPs transplanted without the collagen matrix. They reported that the collagen-NP scaffold promoted tissue repair better than the NPs alone. Yu et al. [55], also reported that some NPs differentiated into neurons and formed synapses, which correlated with improvements in functional recovery. Elias et al. [56], used a similar approach repurposed for TBI. NPs transplanted on the scaffold showed increased survival and migration compared to cells injected without the scaffold; however, neuronal engraftment was not observed as only glial and endothelial cells (ECs) were observed amongst the grafted cells. They concluded that an additional growth factor or biochemical stimulus would be needed to achieve differentiated neurons in vivo. Another popular material being explored is HA, which is an abundant glycosaminoglycan in the brain. Survival rates for each type of precursor cell improved when encapsulated within the hydrogel.

i. *Protein-based biomaterials*

Different methods of purification exist depending on protein desired for scaffold fabrication and animal source. The most commonly used scaffold materials, collagen, can be isolated from a variety of tissues, such as skin, tendon, or bone. In vivo, fibrin, which is derived from fibrinogen, generates the blood clots that form after injury to the vasculature. Due this role and the ability to isolate fibrinogen from blood (both human and animal), it has been used as a sealant in clinical studies and as a biomaterial scaffold. Another protein that has been investigated for use in generating tissue engineered scaffolds is silk, which is secreted by insects and worms. Scaffolds made of silk or silk fibroin

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have slow degradation rates and desirable mechanical properties, providing an alternative to the collagen and fibrin. Scaffolds made from silk fibers can be fabricated into a variety of structures, such as mats, sponges, meshes and membranes, expanding the possible applications. Silk can also be chemically modified to further enhance the properties of such a scaffold.

3D collagen scaffolds have been used to culture a wide variety of stem cells for different TE applications. One study demonstrated the monkey embryonic stem (ES) cells could differentiate into neural phenotypes as well as endothelial phenotypes when cultured as aggregates of cells known as embryoid bodies inside of collagen scaffolds [57]. Additionally, other types of stem cells have been used in conjunction with collagen scaffolds to produce engineered tissues. These approaches include seeding such scaffolds with NSCs [58]. These cells differentiate into neurons and form functional circuits inside of the scaffolds [59]. Although it has not been investigated as heavily as collagen, fibrin has also been studied as potential scaffold material for the culture of stem cells. A variety of stem cell lines can be cultured inside of fibrin scaffolds for many different TE applications. The properties of silk make it attractive for engineering bone, cartilage and ligament tissue and extensive research has been done using 3D silk scaffolds in conjunction with mesenchymal stem cells for these applications.

ii. *Polysaccharide-based biomaterials*

Agarose is isolated from red algae and seaweed, consists of a galactose-based backbone and is commonly used as a medium for cell culture in the form of agar. One of the attractive properties of agarose is that its stiffness can altered, allowing for tuning of the mechanical properties of the scaffold. Agarose scaffold have been investigated in combination with stem cells for generating a variety of applications, including cartilage, heart, and nerve. Studies have demonstrated that both mouse and primate ES cells can differentiate into dopaminergic neurons when encapsulated inside of agarose microcapsules [60]. This strategy could be used as a potential therapy for Parkinson's disease. In nutshell, agarose scaffolds provide a versatile platform for TE.

Alginate, which is derived from the cell walls of brown algae, forms scaffolds through the use of ionic cross-linking, allowing for encapsulation of cells. Alginate has also been used for neural TE applications. One study demonstrated that adult NPCs seeded inside of alginate scaffolds survived in vivo for two weeks after implantation into a spinal cord injury model [61]. A different study developed tunable alginate scaffolds by incorporating microspheres that released enzymes over time to degrade the scaffold. These scaffolds were successfully used to culture NPCs and increased their proliferation rate compared to when such cells were cultured in alginate scaffolds without microspheres [62]. Hyaluronan, also known as HA, is one of the major components of the ECM. It contains sites for cell adhesion and hyaluronan expression is upregulated during embryogenesis, suggesting its suitability as a scaffold material for the culture of ES cells. Hyaluronan is also expressed in many different tissues, including cartilage and nerve, suggesting it could also be used for the culture and differentiation of adult stem cells. Another polysaccharide that has been explored for TE applications is chitosan. It is derived by deacetylation of chitin and consists of glucosamine units. Chitosan has been used extensively as material for regenerating skin, bone and nerve tissue and is recently studied for use in combination with stem cells.

iii. *Natural Surfaces and Gels*

Numerous surfaces and gels have been generated from natural components such as collagen, other ECM proteins, and calcium alginate. However, natural components can face several challenges. It can be difficult to tune the mechanical properties of natural materials, and it is generally not possible to independently tune the mechanical and biochemical signals of these systems. Natural components, such as ECM proteins, also have problems with purity and the availability of large-scale sources of the materials, particularly if human proteins are involved. Numerous efforts have used 3D type I collagen, which can form gels, to culture rat embryonic cortical NSCs [59]. In one study, O'Conner et al. [63] cultured neurospheres on the top of collagen I gels and found that cells were able to migrate and disperse from the spheres and subsequently extend neurite processes. Most cells remained attached to and proliferated on gel surface during first week of culture, and cells that did differentiate during this initial time gave rise primarily to neurons that showed capacity to form synapses. During second week of culture, remaining NSCs differentiated into glial cells [63]. ECM molecules other than collagen have also been used to prepare surfaces for culture and differentiation of NSCs. Results demonstrated that precursor cells propagated with same mitogen can exhibit a different behaviour as a function of substrate.

Neurospheres of postnatal human cortical NSCs and mouse embryonic cortical NSCs have been analysed on various ECM proteins adsorbed to glass surfaces. The R 6 integrin was shown to be functionally important for cell attachment to LN [64]. Studies showed the importance of tuning the mixture of soluble factors and substrates to elicit specific cellular behaviours. ECM and other factors combine to regulate cell behaviour, which raises the experimental difficulty of exploring many possible combinations of factors. However, complex combinations of factors, including ECM, may be necessary to achieve tight control over cell function. Microarrays can yield substantial information on the

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combinatorial effects of substrate and soluble factors on cell function, results that will aid the development of bioactive, synthetic microenvironments. In addition to high-throughput screens, surface patterning can be used to analyse the effects of spatially organized signalling factors on cellular behaviour. Because alginates are both biocompatible and inexpensive, they have been broadly explored in cell encapsulation and tissue-engineering applications [65]. Studies show the potential of calcium alginate for engineering microenvironments for NSCs and results indicate that when encapsulated in some materials, cells can presumably provide their own signals and therefore do not require the addition of ECM molecules, although adding exogenous signals may afford more control over cell behaviour.

iv. *Semisynthetic Surfaces and Gels*

Surfaces and gels have also been developed using a blend of synthetic and natural components. The natural component in these blends is typically an ECM protein that is adsorbed to the synthetic component and presents signals to modulate cell attachment, growth, and differentiation. Moreover, the addition of a synthetic component enables control over the architecture and mechanics of the materials. These bioactive, modular materials can therefore be viewed as an intermediate step toward developing completely synthetic materials, although the ECM protein still poses challenges for purity, immunogenicity, scalability, and other considerations. Studies collectively demonstrate that natural components can provide biochemical signals necessary to support cell attachment, proliferation, and differentiation when presented from a synthetic substrate. Promising semisynthetic materials also provide a promising basis for the development of fully synthetic materials that avoid some challenges of using isolated proteins, as these can potentially be replaced with recombinant or synthetic signals.

v. *Fully Synthetic Surfaces and Gels*

Natural ECM proteins offer the important advantage of presenting both identified and likely unidentified motifs that bind to cellular receptors and thereby regulate cell behaviour. However, natural components have the potential to elicit an immune response if implanted, can transfer immunogenic molecules to stem cells, [66] can pose a risk of pathogen transfer, and often do not offer the capacity to readily control the mechanical properties of the material. By comparison, materials composed of primarily synthetic components offer advantages including low immunogenicity, reproducible and scalable synthesis, and the ability to tune mechanical and biochemical properties, an important consideration for stem cells [53].

vi. *Self-Assembling Peptides and Peptide Amphiphiles*

Specific polypeptide sequences have the capacity to self- assemble into various structures, ranging from assembly of β-sheets via hydrogen bonding to cylindrical micelles via hydrophobic interactions [67]. To build upon these capabilities for creating bioactive matrices, self-assembling peptide sequences can be synthesized as fusions to motifs found in ECM proteins, including RGD and IKVAV from fibronectin and LN [68] respectively to create self-assembled structures that can engage cellular adhesion receptors. These synthetic peptides also offer the advantage of being able to display a broad diversity of natural and even unnatural side chains from the peptide backbone, enabling creation of multifunctional assemblies. A study using peptides that assemble into fibrous structures via β-sheet formation showed that this scaffold encouraged putative neural stem or precursor cells from adult rat hippocampal slices to migrate away from tissue explants laid on top of the scaffold [69].

c) *Synthetic biomaterials*

Although not as commonly employed as natural materials, synthetic materials also have been used in brain TE applications. Bible et al. [70] determined that transplanting MHP36 NPs into intact tissue lead to further damage. Some groups are encapsulating NPs into self-assembling peptide hydrogels. Peptides readily self-assemble and they can form nano-fibrous networks that mimic native ECM. Moreover, like hydrogels they can be injected in soluble form and subsequently solidify to form gels in situ. Li et al. [71] reported utilization of graphene foam, a 3D porous structure, as a novel scaffold for NSCs in vitro. It was found that 3 graphene foams can not only support NSC growth, but also keep cell at an active proliferation state with up regulation of Ki67 expression than that of 2D graphene films. 3D-GFs can enhance the NSC differentiation towards astrocytes and especially neurons.

i. *Polymer-based biomaterials*

The Polymer-based scaffolds have specific mechanical properties and can be modified to contain cues using various chemistries. There are some issues with these polymer-based scaffolds including a lack of sites for cell adhesion and the potential for toxic by-products after degradation. Poly (lactic-co-glycolic acid) (PLGA) is a copolymer that consists of monomers of glycolic acid and lactic acid connected by ester bonds. Neural TE represents another area where PLGA scaffolds seeded with stem cells shows promise as therapy for disorders of the nervous system. Work done by the Langer lab has shown the potential of such strategies. One study that showed that PLGA scaffolds designed to mimic the spinal cord and seeded with murine NSCs produced an increase in functional recovery after traumatic spinal cord injury in preclinical testing [72]. An additional study demonstrated that

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human ES cells seeded inside of PLGA scaffolds could be directed to differentiate into neurons when treated with the appropriate cues [73]. The same study also showed that these cells could differentiate in cartilage and liver tissue inside of such scaffolds when exposed to the appropriate cues. A follow up study further characterized the differentiation of human ES cells treated with neurotrophins when seeded inside PLGA scaffolds for engineering neural tissue [74]. Seeding retinal progenitor cells into PLGA scaffolds provided an effective method of cell delivery in vivo, and the cells were able to differentiate into neurons and astrocytes [75]. PLGA has also been demonstrated to be a suitable scaffold for the culture of progenitor cells isolated from the hippocampus in terms of cell viability and differentiation [76].

Poly (ethylene glycol) (PEG), with high molecular weight versions being referred to as poly (ethylene oxide), is a commonly used polymer for biomaterial applications due to its ability to resist protein absorption. Other examples in the literature show the suitability of PEG scaffolds for engineering nerve tissue for the treatment of CNS disorders, such as Parkinson's disease or spinal cord injury. Work by Mahoney and Anseth demonstrated that NP cells could be cultured inside of PEG scaffolds and investigated the effects of adding bFGF (basic fibroblast growth factor) and collagen to such a system [77]. PEG scaffolds functionalized with poly-L-lysine to add sites for cell adhesion, and the NSCs seeded inside these scaffolds survived and were able to differentiate into mature phenotypes [78]. They used macro porous PEG scaffolds for the co-culture of NPCs and ECs to engineer nerve tissue. The addition of the ECs allowed for formation of a microvasculature inside of the nerve tissue when tested in vivo [79].

ii. *Peptide-based biomaterials*

Peptide-based biomaterials consist of short sequences of amino acids, which can produce self-assembling scaffolds. These scaffolds can potentially combine the functionality of protein-based scaffolds by using motifs derived from naturally occurring proteins with the reproducibility of synthetic scaffolds. Many of the peptide-based biomaterials can self-assemble into 3D scaffolds through the use of amphiphilic peptides, which form aggregates in aqueous solutions. The Stupp lab was one of the first groups to use such self-assembling scaffolds for promoting the differentiation of murine NPCs into neurons [67]. These scaffolds contained the peptide sequence IKVAV derived from LN and this sequence had been shown previously to promote neurite outgrowth [80]. The importance of selecting appropriate peptide sequence for promoting survival and differentiation of stem cells seeded inside of such a scaffold is also illustrated.

iii. *Ceramic-based biomaterials*

Ceramics are inorganic materials formed through treatment with heat and are often porous and brittle. They have crystalline structures and are used for a wide variety of applications.

iv. *Synthetic Polymers*

NSCs have also been cultured on numerous synthetic polymers, many of which have previously been used with other cell types for many applications including TE and controlled drug delivery [81]. Optimizing these materials may lead to the development of reproducible, scalable, nontoxic, and nonimmunogenic materials for in vitro expansion or differentiation, as well as in vivo implantation, of NSCs. In summary, fully synthetic, bio functionalized materials can support cell proliferation, and the addition of differentiating media leads to multipotent differentiation. Future work may explore the extent to which the substrate can guide cell lineage commitment. Furthermore, the use of thick gels can enable studies of the effects of matrix mechanics on NSC proliferation and differentiation [82].

d) Incorporating growth factors

Many of the aforementioned studies reported that cell survival was often poor and neuronal differentiation difficult to achieve from transplanted neural stem and progenitor cells. Therefore, investigators have found that they need to increase the complexity of their scaffolds to incorporate survival and/or differentiation factors. Neurotrophic factors have been incorporated into biomaterial based drug delivery systems to promote nervous tissue repair. For the past several years scientists have endeavoured to produce a multifunctional microsphere scaffold optimized for transplanting NPs into the TBI brain. NPs transplanted without a scaffold often remain clustered at the site of injection [83]. It is possible that the Fibroblast Growth Factor-2 that is delivered on the scaffold is facilitating the migration of the NPs [84]. Several of the groups listed above also have reported greater migration of transplanted NPs when delivered using a biomaterial scaffold.

e) Type of Biomaterial Scaffold Structures

From this list we may narrow down the types of scaffolds and the compositions of biomaterials optimal for use. Since an injectable scaffold is desired, this significantly limits the biomaterials available. Two common designs that would apply would be hydrogel systems and micro or nanoparticle systems. Hydrogels are liquid, but undergo gelation upon injection into the brain. Often times this is achieved through the change in temperature from ambient air temperature of ~ 21°C to the body temperature of ~ 37°C. Alternatively, micro or nanoparticles could be produced varying in configuration from microscopic spheres, irregular

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particles or as fibers that are subsequently suspended in a liquid or gel for transplantation.

i. *Hydrogels*

Hydrogels are water-soluble polymer chain networks. They can absorb up to 99 percent water, which makes them a strong candidate for brain scaffolding. They have excellent nutrient and oxygen permeability, allowing cell survival in the scaffold [85]. Hydrogels can also be modified with proteins, GAGs, cytokines, drugs and other factors that will stimulate cell adhesion and/or growth [86]. Cells are readily encapsulated into hydrogels to replace missing autologous cells. Most importantly, hydrogels form in situ. As their name suggests, they gel following injection into tissues [87]. Furthermore, hydrogels possess elastic properties that are similar to those of natural brain tissue. Hydrogels can be created with low compressive moduli that tend to direct stem cell differentiation toward neural lineages [88]. A downside to hydrogels is that cellular migration and outgrowth is often poor due to its weak mechanical structure. In the CNS migration is essential for the initial formation of cortical architectural, for axonal growth and synaptogenesis and for white matter colonization by oligodendrocyte progenitors prior to myelination. Moreover, cells, and in particular neurons, do not extend their neurites through three-dimensional matrices efficiently [89]. Neurite outgrowth is best observed on 2-D rigid structures. This is due, in part, because neuronal growth cones require stiff substrates to pull on in order to grow or stretch. The filo podia of many cells have similar properties. Cells placed onto softer substrates are often round and maintain very short processes. Thus a hydrogel will not likely create a suitable environment for radial glial cells that naturally extend their processes long distances to the pial surface of the brain during embryonic development. Another disadvantage in using hydrogels is that their biodegradation is hard to control [90]. Because the majority of hydrogel systems focus on gelation and cytocompatibility, degradation rates are often sacrificed or difficult to manipulate.

ii. *Microspheres and micro particles*

Microspheres and micro particles on the other hand, possess a rigid surface structure, as opposed to the soft structure of hydrogels. Due to their rigidity, the tension that neuronal growth cones require can be created and maintained more easily on microspheres than on hydrogels. Furthermore, microspheres can be transplanted by syringe, whereupon they can mould to the injury dimensions. In addition, microspheres can be fabricated to encapsulate, immobilize and deliver specific growth or trophic factors to aid engraftment and survival of the transplanted cells [91]. A downside in using microspheres is that they may be more difficult to inject than hydrogels, since hydrogels are liquid within the injection syringe and gel upon contact with the brain (usually due to temperature differences) whereas, micro particles typically need to be suspended in an additional solution. Another limitation is the weak elasticity of micro particles. Stiffness might increase neurite outgrowth, although it might also decrease differentiation. Studies have shown that materials constructed with elastic properties similar to that of natural brain tissue are more likely to favour neuronal differentiation [92]. Microspheres are inferior in this regard.

V. Biomaterial Parameters for Directing Neural Stem Cell's Fate

The natural NSCs niche provides a model for designing a powerful artificial microenvironment to regulate the NSCs fate, which is essential for the CNS regeneration (Fig. 3). The cells, blood vessels, and the ECM in the NSCs niche work together to determine the fate of NSCs [5]. According to their different properties, biophysical and biochemical parameters can be concluded as two main stem-cell-regulatory cues in the NSCs niche. The biophysical parameters contain the mechanical properties and architecture of the ECM. The biochemical parameters are composed of the chemical and bioactive cues originating from the soluble cytokines and growth factors released by the adjacent cells, cell adhesion molecules, and ECM molecules. A functionalized scaffold for CNS TE and regeneration should be designed to bio mimic the NSCs niche to regulate fate of NSCs.

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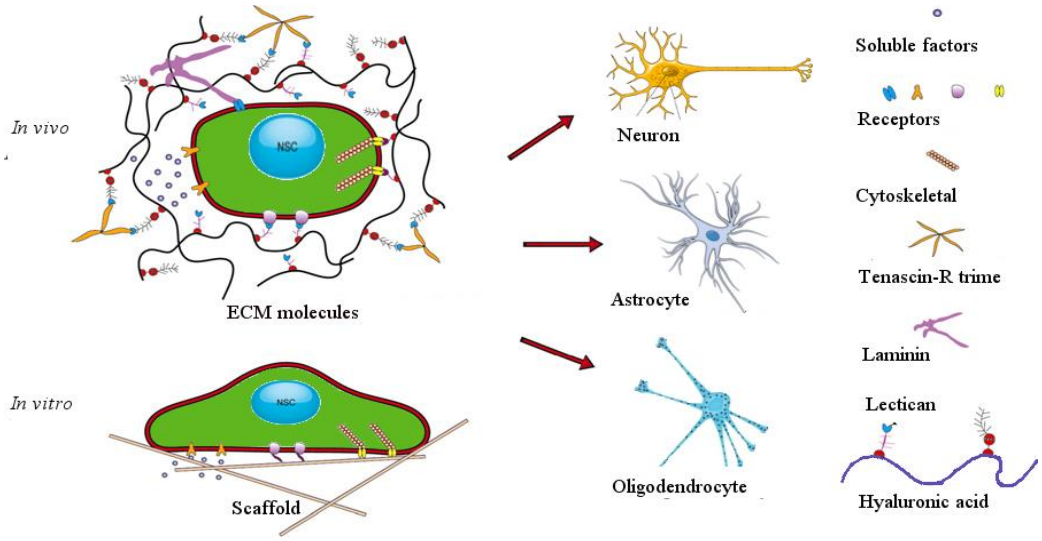


Figure 3 : NSC's Niches (in vivo or in vitro) Directing its Fate for CNS Regeneration

a) Biophysical parameters of designed biomaterials

During the development and throughout the life, the NSCs may be exposed to a variety of biophysical signals, such as tensile, compressive, shear, osmotic, fluid stresses, and so on so forth [93], that often directly provoke the dynamically remodelling of NSCs cytoskeleton networks. The topography and the mechanical property of the substrate are two major biophysical cues influencing NSCs state. For example, tension of the ECM can induce stretch of the cytoskeleton and nucleus through focal adhesions, while compression of the ECM can significantly alter local charge density and ion concentrations, potentially activating osmotically sensitive ion channels [94]. The topography of the substrate provides geometric cues to NSCs in the form of fiber diameter, length, and aligned/interwoven patterns, as well as surface micro/Nano topography. The cell shape was changed in response to different topography, which in turn influenced cellular signalling path-way and cell functions. Similarly, during the cell culture of NSCs in vitro, the biophysical property also momentously affects the adhesion, differentiation and proliferation of the NSCs [89].

i. *Topographic cues of the substrate*

The well-defined matrix architecture of stem cell niche, such as unique nano-fibrous characteristics of basal lamina membrane in different tissue relates to the specialized cellular functions, suggesting the importance of substrate topography in stem cell niche [95]. Previous studies demonstrated that micro-to-nanoscale substrate topography plays an important role in controlling the adhesion, proliferation and differentiation of NSCs. For instance, the rat NSCs (rNSCs) were cultured on polyether sulfone fiber meshes with average fiber diameter of 283 nm, 749 nm and 1452 nm respectively under the differentiation condition (1 mm retinoic acid and 1% foetal bovine serum) [96]. It was shown that rNSC had an oligodendrocyte differentiation on smaller fiber mesh (283 nm) and neuronal differentiation on larger fiber mesh (749 nm) compared with tissue culture plate. And the rNSCs showed lower viability on fiber mesh with diameter 1452 nm. Besides, when the rNSCs were cultured in serum free medium, higher degree of proliferation and cell spreading and lower degree of cell aggregation were observed as the fiber diameter increased. Besides the fiber diameter, the aligned substrate topography has also been proven to influence NSCs morphology and neuronal differentiation [97]. The random and aligned Polycaprolactone fibers with average diameter of 260 nm, 480 nm and 930 nm were yielded by electrospinning. NSCs elongated along the major fiber axis and a higher fraction of cells exhibited neuronal differentiation marker (Tuj1) compared with random matrix of similar dimensions. Aligned fibers could guide Tuj1þ cells to extend neurites several times of the length of the cell body along the axis of fiber alignment. While, such cells on the random fiber meshes showed randomly extended neurite pattern. Among the fiber meshes, 480 nm diameter aligned fibers supported highest fraction of neuronal differentiation.

In addition to the geometrical features at the nanoscale level, the higher level of organization of the substrate was also proven to be important for cell growth. Three kinds of chitosan scaffolds with different topologies (film, porous scaffold and multi microtubule conduit) were introduced to influence the fate of rat NSCs [98]. In the presence of Foetal bovine serum, chitosan film supported rNSCs to differentiate more easily into astrocytes, while rNSCs preferred to differentiate into neurons in chitosan multimicrotubule conduit and porous scaffold. In serum free medium with 20 ng/ml bFGF, rNSCs showed an increased

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differentiation trend on all the types of chitosan scaffolds.

ii. *Mechanical properties of the substrate*

NSCs may encounter different mechanical microenvironments in adult brain, including blood vessels, layered cells structure, glial scars and grey and white matter, which may present variable modulus (#10 2 –10 3 Pa) [99]. Recent work indicates that primary neural cells exhibit mechanic-dependent neuronal morphological differentiation and glial survival [100]. NSCs differentiation into neurons increases when they are cultured on softer substrates with modulus similar to that of native brain. Saha et al. [82] developed a hydrogel culture system to assess the adhesion ligand presentation and material modulus from 10 to 10000 Pa on adult NSCs behaviour. In serum-free neuronal differentiation medium, scaffold with the physiological stiffness of brain tissue (500 Pa) favoured NSCs to differentiate into neuron. Under the mixed differentiation medium, soft gels with modulus of #100–500 Pa greatly favoured neurons, whereas harder gels with modulus of #1–10 kPa promoted glials. Besides, substrates with modulus of #10 Pa inhibited cell spreading, self-renewal and differentiation. Similar phenomenon was also observed on the behaviours of encapsulated NSCs in different mechanical properties of alginate hydrogels [92]. Different alginate hydrogels with modulus from 180 to 20000 Pa were obtained by varying the concentrations of alginate and calcium chloride. The rate of proliferation of NSCs decreased with the increase of the modulus of alginate hydrogels. The softest hydrogels which had similar modulus of brain tissues greatly enhanced the expression of neuronal marker b-tubulin III.

In addition to moduli of biomaterials, neural crest stem cells (NCSCs) were subjected to cyclic uniaxial strain to determine whether vascular mechanical strain modulated the differentiation of NCSCs into smooth muscle lineage [101]. Mechanical strain enhanced NCSCs proliferation and smooth muscle cell differentiation, and suppressed the differentiation into Schwann cells (SCs). Besides, sinusoidal inertia force (at 12.5 Hz, 25 Hz or 50 Hz of frequency, and 0.25 G or 0.5 G of acceleration amplitude) was also applied to cultured NSCs and could have effects on NSCs [102]. The mechanical vibration at 25 Hz is most effective on cell proliferation at 0.25 G. The enhancement of cell proliferation is probably caused by the suppression of apoptosis. The differentiation of the NSCs depends on acceleration amplitude and the mechanical vibration may maintain some properties of stem cells.

b) Biochemical parameters of designed biomaterials

In the NSCs niche, lots of biochemical factors work together or signally regulate the fate of NSCs. Recently, many in vitro studies showed that the NSCs were sensitive to their surrounding biochemical factors, such as different surface chemical groups and bioactive cues. NSCs exhibited morphological changes in response to different chemical groups at single cell level. In the downstream differentiation, –SO3 H favoured NSCs to oligodendrocytes, while –COOH, –NH2, –SH and –CH3 support the cells to differentiate into neurons, astrocytes and oligodendrocytes [103]. A type of mouse NPCs were also used to evaluate the effect of different chemical groups on cell behaviours and functions.[6] The chemical functional groups of –N2, –COOH and –SH could promote the secretion of glutamate decarboxylase.

Water soluble factors as a kind of small molecule proteins play an essential role in neural development, differentiation, survival, regeneration and function in both in vivo and in vitro [104]. Stem cell characteristics such as self-renewal and differentiation potential could be maintained by fibroblast growth factor (FGF). Neural and astrocytic differentiation can be induced by PDGF and cardiotrophin-1/ciliary neurotrophic factor, respectively [105]. Oligodendrocytic differentiation can be induced by thyroid hormone, T3. In addition, NSCs can also differentiate to mesenchymal cell lineages with the stimulation of bone morphogenic protein-4 [106]. Similarly, inhibitory factors produced by the reactive astrocytes at the site of injury could inhibit neurite extension outgrowth [107]. Scar or lesion-associated inhibitors encompass CSPGs, myelin associated glycoprotein and members of the ephrin and semaphorin families [108]. Nogo A is up-regulated or accumulated at the human lesion sites [109] and enhances the cognitive defects after experimental brain injury in rodent. Other myelin-associated molecules with neurite growth inhibitory activity play important roles in early development of neuronal maps [108]. To reduce the effect of inhibitory factors in neurogenesis, antibodies of these inhibitors were used to modify scaffold or injected into lesion sites directly. Nogo-66 receptor antibody was used to modify the HA scaffold to block Nogo-66 and simultaneously inhibit the formation of glial scar [110].

In the NSCs niche, the ECM is another vital factor for NSC growth and differentiation, in which intrinsic organization is necessary for influencing NSCs to guide restructuring in host tissues [111]. The neurotransmitters; acetylcholine [112] and dopamine [113] are also reported as biochemical cues of NSCs niche. Collagen is one of the most prevalent ECM molecules in human and other mammalian tissues [114] and is commonly used for immobilization of NSCs [115]. The successful culture of NSCs in three dimensional (3D) collagen gels was previously reported [59]. Little et al. [116] has summarized the use of collagen gels with NSCs in an excellent review. Neurosphere-forming NSCs had good survivals and proliferations in collagen hydrogels with addition of epidermal growth factor [115], and a part of the cells differentiated into neuronal

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and glial lineage. A layer-by-layer printing of double-layered 3D collagen scaffold with NSCs and VEGF-containing fibrin gel was introduced to study changes of murine NSCs morphological and migration [117]. Cells migrated towards fibrin gel with VEGF and showed growth factor-induced morphological changes.

HA is another essential organization and structural component in the ECM of native tissues [118]. HA is particularly abundant in the foetal brain and surrounds immature neurons during differentiation in the spinal cord [119], which has been shown to significantly influence nerve regeneration, neuronal and glial development [120]. Recently, NSCs were photo-encapsulated into HA hydrogels and remained viable after encapsulation [88]. HA modified with polylysine and anti-NgRs were developed as scaffold for both neurospheres and single NSCs [121]. After 5 days cell culture, single dispersed NSCs were observed to differentiate into neurons and astrocytes, while neurosphere-forming NSCs migrated from their original aggregate and maintained the NSC phonotype. Incorporation of PLGA microsphere encapsulating brain-derived neurotrophic factor was further explored to promote NSC adhesion and proliferation [122]. NSCs would differentiate into neurons and astrocytes, and neurites extended along the wall of scaffold and formed extensive network. Other natural polymer biomaterials, such as alginate [123] gelation, chitosan and fibrin [124], have also been used to prepare 3D culture models for NSCs/NPCs. Alginate composition (the ratio of D - mannuronic and L -guluronic acid) will affect the NSCs survival and proliferation [123]. Fibrin will support NSCs to neuron differentiation and inhibit proliferation of astrocytes [124].

Although natural scaffolds offer important advantages for cellular receptors binding to regulate cell fate, they may transfer pathogen immunogenic molecules to stem cells [66]. In comparison, synthetic poly-peptide composed of biological building blocks offer advantages including none or little immune response, reproducible and scalable synthesis, and amenable to design and modification to achieve specific needs [125]. Many types of self-assembling peptides are designed to undergo spontaneous assembly through weak interactions into well-ordered interwoven nanofibers in water and rapidly form a gel-like 3D network, which is similar to the structure of natural ECM [126]. Since the building blocks are natural L -amino acids, these peptide scaffolds are chemically compatible with aqueous solutions and physiological conditions. Most importantly, specific cell interaction bioactive motifs could be conjugated into the peptides to enhance their interaction with NSCs. For example, IKVAK motif has been shown to encourage differentiation of NSCs into neurons [67]. RADA16-I is one of such poly-peptide biomaterials, which contains alternating amino acids that contain 50% charged residues [127]. The peptide could undergo spontaneous assembly into well-ordered interwoven nanofibers in water and rapidly form hydrogel with #10 nm fiber diameter, 5–200 nm pore size, and over 99% water content under physiological conditions.

VI. In Vitro Testing of Biomaterials to Improve Cell Survival

Extensive in vitro studies have developed 2 D surfaces or 3D gels for culturing either relatively uniform NSC populations or to a lesser extent CNS tissue explants. These efforts have focused on engineering substrates, sometimes in conjunction with growth or other soluble factors, which support or regulate specific cellular behaviours such as proliferation, differentiation into either neurons or glia, or neurite growth from neurospheres. The development of materials for in vitro cell culture is important for stem cell expansion and differentiation and can also serve as a first step towards design of materials that can support the survival and engraftment of stem cells in vivo upon implantation. Numerous studies have been performed in vitro to compare the efficacy of scaffolds for neuronal differentiation and survival [128] reporting the efficacy of stem cells transplanted together with a biomaterial matrix in TBI models.

VII. Mechanisms of Cell Death Following Cell Transplantation

Many factors contribute to cell death following cell transplantation including: time after injury; [129] distance from the transplantation site to the epicentre of injury; 10 state of the cells transplanted—differentiated or undifferentiated; [130] developmental state of cells transplanted—embryonic versus adult; [131] mode of cells delivered—single cells vs. neurospheres; [132] host immune response; [133] and phagocytocytic response of host [134]. The mechanisms of cell death following transplantation were investigated by Hill et al. [135]. The percent of surviving cells was found to be consistent irrespective of the number of cells injected. Necrosis was the leading cause of death for transplanted cells during the first 24 hours after transplantation, resulting in 6 times more cell death than apoptosis. Since apoptotic SCs diminished after the first 24 hours following transplantation, the authors postulated that apoptosis may have been initiated prior to transplantation in response to removal of serum, mitogens or ECM. During cell culture prior to transplantation, there are two main mechanisms that contribute to cell death: detachment of cells from their adherent surface and the removal of optimal growth factor concentrations. Therefore, when cells are prepared for transplantation as single cells, integrin–ECM interactions are lost and apoptosis is initiated. Cell

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survival is further limited by the additional cell death induced by the environment at the injury site.

a) In vitro testing of biomaterials to improve cell survival

To increase survival, cells have been delivered in biomaterial scaffolds that are designed to provide the cells with a permissive microenvironment. This microenvironment includes chemical and physical cues designed to guide cell growth and integration with the host tissue [136]. In order to identify a suitable biomaterial for cell delivery, it must be first tested for cytotoxicity. For example, Puramatrix, which is a peptide hydrogel, was found to be cytocompatible at 0.25% but cytotoxic at 1% to human foetal NSCs, demonstrating that gel concentration is as important as gel composition [137]. Importantly, the effect observed with NSCs may be different for another cell type or even the same cell type from another species, thus the biomaterial has to be designed and tested for a specific cell type and injury. Combination strategies of biomaterials and growth factors have been studied for cell delivery. It is well understood that cell survival is improved in the presence of growth factors. However, when designing a biomaterial it is important to consider how the growth factors can be co-delivered with the transplanted cells to provide a sustained and localized release.

Synthetic materials such as PLGA have also promoted cell survival in vitro cultures of neural cells. PLGA has been investigated as it has good biocompatibility; is easily manufactured; and is believed to reduce scarring and cyst formations in models of SCI. NSCs grafted into PLGA slices of 2 mm depth were viable after 14 days of culture [138]. Electrospun poly (3-caprolactone) (PCL) nanofiber scaffolds promoted the in vitro survival of cortical cells. Similar to PLGA, PCL is biocompatible and has been investigated as a biomaterial to increase cell survival. Electrospun nanofibers can be modified to control the fiber alignment, diameter of the fibers and interfiber distance. Due to these tuneable parameters, it is proposed that electrospun nanofibers can provide a 3D environment to stimulate neural cells. To maintain a local supply of BDNF, PCL scaffolds were chemically modified with BDNF. Significantly greater cell survival was observed on PCL scaffolds immobilized with BDNF vs. PCL scaffolds with soluble BDNF or PCL scaffolds alone. However, despite increased cell survival, the proportion of apoptotic cells was not significantly reduced compared to 2D culture on PDL-coated glass coverslips [139]. While chemical modifications of scaffolds with growth factors can improve cell survival, methods to decrease cellular apoptosis on scaffolds must also be addressed. Poly (D-lysine) (PDL) is known to attract neurons and promote neurite outgrowth, and for this reason it has been used in numerous cell culture experiments. While the interaction with neurons is non-specific, PDL provides generically cell-adhesive substrates.

b) Improvement of cell survival using biomaterials in vivo

In vivo studies shows the promise of PGA, PLGA, and alginate materials in TE for the spinal cord and brain. Donor NSCs were able in some cases to aid in recovery from the injury and differentiate in vivo into different proportions of glial and neuronal cells. In addition, these observations were dependent on the chemical microenvironment created by the material, as well as its topological structure. Furthermore, host neuronsand glial cells were even able to incorporate into one of the scaffolds. Results were generally better when both the cells and the scaffold were used, showing the combined promise of biomaterials and NSCs in tissue regeneration.

Transplantation of alternative cell types has been proposed as a method to increase cell survival. Immune rejection decreases cell survival after transplantation. To minimize immune rejection of transplanted cells, the immunosuppressant cyclosporine was co-delivered with SCs [133] or neural stem progenitor cells (NSPCs) [140]. Co-delivery of 10 mg kg ῖ1 and 20 mg kg ῖ1 cyclosporine, respectively, enhanced cell survival in both cases; however, prolonged immunosuppression is problematic for the patient's overall health and thus this strategy is limited. While the ultimate goal of designing biomaterials in nerve regeneration is to control the endogenous and exogenous NSCs in vivo with the biomimetically artificial niche and achieve complete functional recovery of injured nerves. These extracted biophysical and biochemical parameters will actually elicit synergistic effects on directing NSCs lineage commitment, as well as the behaviours of many other types of tissue cells. More than that, peptide amphiphiles were another type of impressive synthetic polymers for nerve TE. Diblock copolypeptide amphiphiles have great promise as highly versatile and finely tunable hydrogels for potential therapeutic applications in CNS regeneration.

As reported that the SVZ was rich in a plexus of blood vessels that snaked along [141], and NSCs closely apposed to the LN-containing ECM surrounding vascular ECs. It was proved that normal SVZ cells in vivo tend to proliferate adjacent to blood vessels because the ECs can stimulate self-renewal and expand neurogenesis of NSCs by releasing soluble factors. The activated NSCs give rise to transit amplifying cells, which in turn generate neuroblasts in the SVZ niche. Neuroblasts differentiated from NSCs may migrate away from the niches and then underwent differentiation into certain lineages in a specific destination. It has been observed that the complex and far-reaching form of neural migration occurred even in the adult brain [142]. The neuroblasts migrate from the walls of lateral

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ventricles to the olfactory bulb where they differentiate into local interneurons. While it is not very clear what the driving forces of neuroblasts migration away from SVZ are. One of the possible reasons is ependymal flow arisen by the formation of chemo repulsive gradients in SVZ.

Given the limitations of the endogenous NSCs, transplanting exogenous NPs into the injured brain has gained traction as a more appropriate solution to promote CNS regeneration. Yet this raises the issue of which cell type to transplant. Since brain injuries result in the demise of a range of different neuronal cell types as well as the astrocytes and oligodendrocytes that support them, the ideal cell would be one that has the capacity to produce a large repertoire of different neurons and glia. To date, several types of CNS progenitors as well as several NSC lines have been transplanted into the injured brain. Generally speaking, studies that transplanted progenitors or more differentiated cells have been less successful than studies using NSCs in replacing or rebuilding a neural circuit. Although there is no study directly comparing neuron, progenitor and stem cell transplantations, the vast majority of research on CNS regeneration focuses on the use of stem cell or early progenitor therapies. Lineage progression from a stem cell to a mature neuron is a process in which proliferation, migration and multipotential capacity decreases. Bliss et al. [143], transplanted human post-mitotic neurons (from hNT cell line derived from human teratocarcinoma) into a rat model of stroke and noticed low donor cell survival [144]. Although they saw neurite extension from hNT neurons, there was no migration. Poor cell survival in the cell preparation and during the transplantation process has been noted, especially when transplanting more committed cells into the unwelcoming milieu of a focal neocortical injury. Thus stem cell transplantation studies are more commonly observed in CNS therapeutics, whereas neurons and more differentiated cell types are generally avoided. Bone marrow stromal cells have been shown to improve outcome after brain injury and stroke [145], but the evidence suggests that the functional improvements obtained are not a result of cell replacement but are due to secreted factors that are neuroprotective.

These experimental studies suggest that many obstacles have been overcome in the grand quest to heal TBI with exogenous cell transplants, but the extent of neuronal cell replacement has still been variable and few of transplanted cells are retained [146]. Most of the transplanted cells either do not survive [147] or differentiate into glial cells instead of neurons [148]. This is a concern that the stem cells transplanted do not differentiate into reactive astrocytes that can contribute to glial scarring. Shear et al. [149] and Boockvar et al. [150], found that NG2 positive glial cells were produced upon transplanting NPs and Sun et al. [151], observed that the majority of the precursors that they transplanted became Olig2 positive cells (pre- sumably glia). Ma et al. [148], transplanted NPs (comprised of 4% NSCs) and reported that only 11% of the differentiated cells expressed a neuronal marker. Poor survival of NPs were observed when transplanted directly into the parenchyma following TBI [152].

i. *Improvement of cell survival using biomaterials for cell transplantation into the brain*

Synthetic biomaterials have better defined chemical structures and origins than naturally derived materials which can be advantageous. Polyglycolic acid (PGA) was investigated as a scaffold for NSC transplantation into injured brain. NSCs were transplanted alone or on PGA scaffolds (on which they had been cultured in PGA for 4 days) into brain 7 days post injury. The injury induced cavity filled with new parenchyma and there was minimal monocyte infiltration into the NSC–PGA complex at the interface between the complex and the host cortical penumbra whereas there was significant monocyte infiltration in the untransplanted infarct controls. Astroglial scarring was also reduced in PGA–NSC transplanted groups relative to non-transplanted infarcts. It was proposed that the reduction in astroglial scarring was due to either inhibitory factors produced by the NSCs, the mechanical features of the scaffold or the actions of the NSC–scaffold complex upon the host's injury response [111]. To support NSC survival following transplantation into brain, NSCs have been delivered on fibronectin-coated PLGA particles which provide sites for cell adhesion [70].

ii. *Improvement of cell survival using biomaterials for transplantation into the spinal cord*

Non-ECM derived natural materials have also been used in cell transplantation strategies to improve cell survival. Chitosan and chitin films were shown to promote cell survival in vitro [153] and investigated as cell guidance channels to promote survival of transplanted NSPCs. Three million brain-derived or spinal cord derived NSPCs were seeded in chitosan tubes coated with LN and implanted in the injured rat spinal cord after transection of the cord. Brain-derived NSPCs showed a significantly greater survival than spinal cord-derived NSPCs 12 weeks after transplantation, yet the increased cell survival did not translate to improved functional recovery or axonal regeneration [154]. In a combinatorial approach, ECs and NSPCs were co-delivered in a two-component biomaterial composed of an outer PLGA scaffold and an inner PEG/poly-L-lysine macro porous hydrogel to the injured rat spinal cord in a hemisection model of SCI. ECs were included to promote vascularisation within the transplant to increase cell survival. At eight weeks post-transplantation, the number of functional blood vessels at the lesion site for NSPC/EC + implant animals was

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significantly greater compared to the NSPC + implant. Interestingly, the NSPC/EC + implant was the only group that reformed the blood–spinal cord barrier on the lesioned side of the injury epicentre. Surprisingly, increased vascularisation did not result in increased NSPC survival: at 8 weeks post-transplantation, NSPC survival was 8% in the NSPC/EC + implant group vs. 20% in the NSPC + implant group. The authors attributed this unexpected result to the different number of NSPCs originally transplanted. Since NSPCs produce a number of survival factors, [155] which promote cell survival and 4.5 times more NSPCs were transplanted in the NSPC + implant group than the NSPC/EC + implant group, the difference in cell survival may be attributed to the greater number of NSPCs secreting more survival factors [156].

VIII. Conclusions

Advanced biomaterials can provide a more biomimetic micro environment and significantly contribute to impaired nerve repair and regeneration, which have been an indispensable element in CNS regeneration. Although the discovery of NSCs opens the possibility to achieve CNS regeneration, it is still far from successfully clinical applications, since several challenges, such as precise control of NSCs self-renewal and lineage commitment, structural remodelling of differentiated NSCs, neural reconnection, and correct transmission of nerve signals, are still major obstacles to achieving functional recovery. Therefore, recreating NSCs regeneration niche by designing bioactive materials with complexity of biophysical and biochemical parameters is an important and fundamental prerequisite of CNS TE and regeneration. Each single biophysical or biochemical property of biomaterials will have direct regulatory effect on NSCs fate and should be considered when designing the applied scaffolds. Biomaterial scaffolds allow essential growth factors and other beneficial molecules to be delivered resulting in improved NP survival and repair. All above data indicate that this pNE coating can be a powerful tool to broaden the range of material choices for ex vivo expansion of hNSCs, an important goal for cell therapy.

In order to fully repair a brain lesion, the architecture of the regenerated neural parenchyma must recapitulate the structure of the adjacent host tissue. This is especially true in the case of the neocortex, a region of the brain that is frequently damaged by trauma. The neocortex is a laminar tissue with 6 layers where the neurons located within each layer have specific neurochemical properties and they receive inputs from specific brain regions. Moreover, they send their axons to other, highly specified targets. Thus, in regenerating the neocortex, the neurons that reside in the deeper layers of the cortex (layers 5 and 6) cannot be located in more superficial regions (layers 1 through 4), and vice versa. It has been documented that NPs have the ability to sense their surroundings and reorganize to appropriately fit a cortical layer [157], though it is not likely that transplanted NPs will do the same. Therefore, new biomaterial techniques will be required to ensure the appropriate differentiation and location of NPs within the specific brain region of interest. For the neocortex, we can envision creating a multi-layered scaffold, in which the different biomaterial layers govern the migration, differentiation and survival of appropriate laminar neurons. Alternatively, it might be possible to inject a biomaterial that would organize into a gradient and within this gradient, plasmids, proteins or other bioactive molecules would be organized to promote the systematic migration and differentiation of engrafted NPs [158]. Although it may be more difficult to achieve such a highly organized structure as required to repair neural circuits compared to other organ systems, utilizing TE applications to heal the injured brain remains a promising discipline for future studies.

NSCs are very promising for the treatment of neurodegenerative disorders and injuries of the CNS. Engineered materials containing natural and/or synthetic components can support the expansion and potentially in the future induce the lineage-specific differentiation of NSCs in vitro, with a variety of applications ranging from cell replacement therapy to in vitro diagnostics and screens. Furthermore, highly modular systems that enable the independent variation of mechanical and multiple biochemical signals have strong potential for the application of reductionist biology approaches to understand fundamental mechanisms of stem cell behavioural regulation. However, a number of challenges remain in the design of materials that are nonimmunogenic, scalable, mechanically tunable, and bioactive in their presentation of key regulatory signals to cells. Synthetic materials have considerable promise for offering these capabilities, although challenges remain in the development of synthetic analogues of complex biochemical signals such as ECM proteins. If these challenges can be overcome, however, bioactive materials can be designed to present a microenvironment that can not only support cells in vitro but also protect them in the harsh environment of a diseased or injured region of the CNS and thereby greatly aid stem cell-based regenerative medicine.

Although the combination of stem cells and TE is currently in the research phase and still far from clinical application, it has greatly enhanced the possibility of tissue regeneration. However, many different biomaterials such as nano- biomaterials that have not adapted for use with stem cell culture could be studied in near future. Stem cell transplantation presents a viable strategy for the repair of CNS injury. However, following transplantation cell death is prevalent and limits the efficacy of this technique. Two of the factors

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that contribute to poor cell survival are anoikis and growth factor withdrawal. Biomaterials can be modified with cell adhesion proteins or motifs to improve cell attachment and minimize cell death caused by anoikis. Furthermore, survival factors, such as growth factors, can be encapsulated into the biomaterial to enhance cell survival. By using biomaterials to minimize cell death and promote cell integration with host tissue, more regenerative medicine strategies will be successfully translated to the clinic.

VIII. Acknowledgement

The KVPY Fellowship from Kishore Vaigyanik Protsahan Yojana, Department of Science and Technology, Government of India granted to the first author for the period from August, 2013 to July, 2015 is greatly acknowledged for financial support.

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20. Use good quality grammar: Always use a good quality grammar and use words that will throw positive impact on evaluator. Use of good quality grammar does not mean to use tough words, that for each word the evaluator has to go through dictionary. Do not start sentence with a conjunction. Do not fragment sentences. Eliminate one-word sentences. Ignore passive voice. Do not ever use a big word when a diminutive one would suffice. Verbs have to be in agreement with their subjects. Prepositions are not expressions to finish sentences with. It is incorrect to ever divide an infinitive. Avoid clichés like the disease. Also, always shun irritating alliteration. Use language that is simple and straight forward. put together a neat summary.

21. Arrangement of information: Each section of the main body should start with an opening sentence and there should be a changeover at the end of the section. Give only valid and powerful arguments to your topic. You may also maintain your arguments with records.

22. Never start in last minute: Always start at right time and give enough time to research work. Leaving everything to the last minute will degrade your paper and spoil your work.

23. Multitasking in research is not good: Doing several things at the same time proves bad habit in case of research activity. Research is an area, where everything has a particular time slot. Divide your research work in parts and do particular part in particular time slot.

24. Never copy others' work: Never copy others' work and give it your name because if evaluator has seen it anywhere you will be in trouble.

25. Take proper rest and food: No matter how many hours you spend for your research activity, if you are not taking care of your health then all your efforts will be in vain. For a quality research, study is must, and this can be done by taking proper rest and food.

26. Go for seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.



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27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

28. Make colleagues: Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

29. Think technically: Always think technically. If anything happens, then search its reasons, its benefits, and demerits.

30. Think and then print: When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

31. Adding unnecessary information: Do not add unnecessary information, like, I have used MS Excel to draw graph. Do not add irrelevant and inappropriate material. These all will create superfluous. Foreign terminology and phrases are not apropos. One should NEVER take a broad view. Analogy in script is like feathers on a snake. Not at all use a large word when a very small one would be sufficient. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Amplification is a billion times of inferior quality than sarcasm.

32. Never oversimplify everything: To add material in your research paper, never go for oversimplification. This will definitely irritate the evaluator. Be more or less specific. Also too, by no means, ever use rhythmic redundancies. Contractions aren't essential and shouldn't be there used. Comparisons are as terrible as clichés. Give up ampersands and abbreviations, and so on. Remove commas, that are, not necessary. Parenthetical words however should be together with this in commas. Understatement is all the time the complete best way to put onward earth-shaking thoughts. Give a detailed literary review.

33. Report concluded results: Use concluded results. From raw data, filter the results and then conclude your studies based on measurements and observations taken. Significant figures and appropriate number of decimal places should be used. Parenthetical remarks are prohibitive. Proofread carefully at final stage. In the end give outline to your arguments. Spot out perspectives of further study of this subject. Justify your conclusion by at the bottom of them with sufficient justifications and examples.

34. After conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print to the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects in your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form, which is presented in the guidelines using the template.
- Please note the criterion for grading the final paper by peer-reviewers.

Final Points:

A purpose of organizing a research paper is to let people to interpret your effort selectively. The journal requires the following sections, submitted in the order listed, each section to start on a new page.

The introduction will be compiled from reference matter and will reflect the design processes or outline of basis that direct you to make study. As you will carry out the process of study, the method and process section will be constructed as like that. The result segment will show related statistics in nearly sequential order and will direct the reviewers next to the similar intellectual paths throughout the data that you took to carry out your study. The discussion section will provide understanding of the data and projections as to the implication of the results. The use of good quality references all through the paper will give the effort trustworthiness by representing an alertness of prior workings.

Writing a research paper is not an easy job no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record keeping are the only means to make straightforward the progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear

· Adhere to recommended page limits

Mistakes to evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

· Shun familiar wording, don't address the reviewer directly, and don't use slang, slang language, or superlatives

· Shun use of extra pictures - include only those figures essential to presenting results

Title Page:

Choose a revealing title. It should be short. It should not have non-standard acronyms or abbreviations. It should not exceed two printed lines. It should include the name(s) and address (es) of all authors.



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Abstract:

The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing references at this point.

An abstract is a brief distinct paragraph summary of finished work or work in development. In a minute or less a reviewer can be taught the foundation behind the study, common approach to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study, with the subsequent elements in any summary. Try to maintain the initial two items to no more than one ruling each.

- Reason of the study - theory, overall issue, purpose
- Fundamental goal
- To the point depiction of the research
- Consequences, including definite statistics - if the consequences are quantitative in nature, account quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
- As a outline of job done, it is always written in past tense
- A conceptual should situate on its own, and not submit to any other part of the paper such as a form or table
- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else

Introduction:

The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

- Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done.
- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.

- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
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Procedures (Methods and Materials):

This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.



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Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a argument should be. Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly described. Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved with prospect, and let it drop at that.

- Make a decision if each premise is supported, discarded, or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

- When you refer to information, differentiate data generated by your own studies from available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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ISSN 9755896



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