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MGM Institute of Health Sciences (Deemed University) recognizes the urgent need for promoting medical education in the country, so that the quality of life for individuals and community could be improved by promoting health, preventing and curing diseases, advancing biomedical and clinical research and educational programs for tomorrow's physicians and scientists. The University is committed to creativity, innovation and excellence in every sphere of its working. The University will transform lives and serve the society by educating, creating knowledge and putting knowledge to work. In this endeavor the University has launched a quarterly peer-reviewed scientific journal 'MGM Journal of Medical Sciences' to encourage investigators to publish their research findings for wider dissemination with the aim of applying those for the benefit of the society.

The newly launched peer-reviewed quarterly journal would cover full spectrum of the specialties in biomedical and clinical research. Its third issue would be released in October 2014. The journal aims to publish articles arising out of original research, specialized topics, review articles, editorials, and description of new diagnostic and therapeutic techniques and technologies. In addition, the journal will include pictorial reviews, letters to the editors, book review, and notices of meetings and courses. In this endeavor, the journal hopes to provide a forum for the stimulation of new developments, clinical practices and research in the field of health and allied sciences. The salient feature of the journal would be to bring out from time to time special issues focusing on specific themes of national relevance including the outcome of scientific meetings, etc. A section would be devoted exclusively to young researchers and students in order to encourage them to publish their innovative ideas and research findings. **In fact, it will be a 'student friendly' journal.**

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Editorial

'Teaching is not just another profession. It is a divine responsibility to guide and enlighten', is said very rightly by our honorable Prime Minister of India, Shri Narendra Modi on Teachers' Day eve on 5th September, 2014. Teaching is one of the noblest professions. Teachers imbibe in themselves the qualities of perseverance, integrity and dedication towards the profession of teaching, by virtue of which they are held in high esteem not only by pupils but also by the entire society. An example is Dr Sarvepalli Radhakrishnan, a great revered academic philosopher and scholar. When he became President of India and some of his well wishers expressed their desire for celebrating his birthday, he replied 'instead of celebrating my birthday, it would be my proud privilege if 5th September is observed as Teachers' Day'. The keynote of our culture is Acharya Devo Bhava. The scriptures tell us that if you see your Guru and God together, then fall at your Guru's feet first. This is because your Guru shows you the way to God. And this is why the word 'guru' means 'remover of darkness'. It is the teacher who through intelligence, patience and wisdom polishes the pupil's intellect and aptitude and shapes their bright future, and that is what should be celebrated.

Teacher is a multifaceted personality, a visionary skilled in wide range of teaching approaches such as deep knowledge and understanding; inspiring, motivating and engaging; friendly and helpful; good and effective in communication; sound planning and organizing capacity; and knowledgeable about other material resources that transpire in the classroom and during the teaching-learning processes. To be a teacher, one requires a blend of all these essential attributes to deal with academic needs and upbringing requirements of pupils of all age groups. In addition, teacher is an initiative driven individual, passionate about the role of teaching in shaping the students with diverse backgrounds. Most importantly, they inculcate positive thinking in the minds of students.

The contributions of teachers are to (i) stimulate the academic environment for promotion of quality in teaching-learning and research; (ii) encourage self-evaluation, accountability, autonomy and innovation; (iii) undertake quality-related research studies, consultancy and training programs; and (iv) promote collaboration with other stakeholders of higher education for quality evaluation, promotion and sustenance are highly commendable and appreciated not only by students but entire community. Our teachers are also instrumental in contributing to national development, fostering global competencies among students, promoting the use of technologies and quest for knowledge. They are truly the friend, philosopher and guide to their pupils. Institutions should ensure that the services of the teachers are well appreciated and are provided with adequate opportunities to enhance their skills for advancement of education.

We salute all teachers, who tirelessly light the lamp of knowledge.

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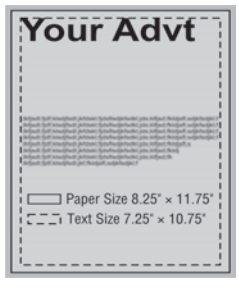

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ORIGINAL ARTICLE

Types of Rotavirus Causing Acute Diarrhea among Children in Western India, their Demographic Pattern and Disease Severity

[1]NC Mohanty, [2]N Agrawal, [3]NN Kadam, [4]A Shamim, [5]M Thakur

ABSTRACT

Background: Rotavirus infection is a major cause of severe acute gastroenteritis among infants and children all over the world[1] with winter out-breaks of diarrhea in temperate and cooler parts almost round the year. However, this varies in different part of India.[2-6] Diarrhea is a major cause of under-5 mortality, contributing to approximately over 1,50,000 infant deaths in our country per year.[15,16] Different genotypes have been identified and many more are emerging by way of mutation, genetic shift and genetic drifts. Rotavirus are classified antigenically as A (Most common), B, C, D, E by ELISA and genotypically as G (1through 12) and P (1 through 8) by Reverse Transcriptase PCR, in combinations.

Materials and methods: Stool samples of 110 infants and children from 6 to 60 months of age, with suspected viral diarrhea over one year period were studied for serotypes and genotypes; and compared for their respective disease severity.

Results: Thirty-four percent were found positive for Rotavirus-A by ELISA. Of the positive, 33.4% were found to be of G9 genotype, much higher than reported from other parts of the country. On the other hand, merely 13.6% of G1 and G4 each were detected, contrary to high prevalence elsewhere. On electropherotyping, the long-arm types were associated with more severe disease (64.6% showing moderate to severe dehydration) than their short-arm types (Only 16.6% showed moderate dehydration only) $p < 0.009$. No difference in incidence of severe dehydration between AD positive for Rotavirus (11.7%) and those found negative (11.8%), presumably due to other viruses, after excluding invasive diarrhea.

Conclusion: Emergence of diverse strains, i.e. more of G9 and G12 genotypes than earlier reports of G1 and G2 types indicate considerable genetic shift in the region. Such trend could have significant implication on degree of seroconversion from currently used live vaccines, using G1 or bovine reassortant G1-3 strains only, seen in recent studies from Africa and Malayi.[29] Contrary to claims that Rotavirus diarrhea usually threatened severe diarrhea, no significant difference in incidence of severe diarrhea was observed between Rotavirus positive and Rotavirus negative acute diarrhea.

Keywords: Diarrhea, Dehydration, Rotavirus, ELISA, Reverse Transcriptase PCR, RNA PAGE (Electropherotyping).

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INTRODUCTION

Diarrhea is an important public health problem in the tropical countries, especially in developing countries like India. It is a major cause of infantile morbidity and mortality world-wide.[1-6] Factors, such as humidity, temperature, climate, sanitation and socioeconomic conditions, contribute to this in a major way.[7,8] Acute diarrhea may be caused by bacteria, virus or parasites. A great majority of cases are due to viruses (Rotavirus 10-35%, Norovirus 2-20%, Astrovirus, Adenovirus 2-10%, Calcivirus, Corona virus, Norwalk virus, etc). Viruses are responsible for more than half of all diarrheas during infancy.[9] In another 45 to 60% cases, no causal agent is detected, possibly due to untypable viruses.

The name Rotavirus is coined from the Latin word Rota (Meaning wheel), because the virus has a distinct wheel like shape. The genome of rotavirus consists of 11 segments of double stranded linear molecules of RNA which are 18,555 nucleoside base pairs. The RNA is surrounded by a double icosahedral protein capsid. The viral particle measures 60 to 80 nm in diameter and is not enveloped.

One of the unique features of rotavirus is its genetic, antigenic and geographical diversity, making it difficult to design a universal effective vaccine. But a sound knowledge regarding types and subtypes of rotaviruses circulating in different regions of India would help in understanding the basis for efficacy or nonefficacy of vaccines which have been designed against this virus.[10]

Reassortment of various rotavirus strains is an important mechanism for generation of their novel and unusual strains. A significant number of children also have mixed rotavirus

infections. There are very few studies in terms of different antigenic and genetic variants from various regions of India so far.

MATERIALS AND METHODS

A total of 110 stool samples were collected in a sterile container from children having acute diarrhea (As defined by WHO) aged between 6 and 60 months, attending pediatrics outpatient Department at MGM Hospital, Kalamboli, Navi Mumbai from 1st July 2009 to 31st December 2011. Presence of tenesmus, frank blood in stool or pus cells in sheets or more than 10 per high power field on microscopy were excluded, presuming these to be of bacterial etiology.

Assessment of Severity

Proper history taking and detailed clinical examination was carried out. The severity of diarrhea was assessed with Vesikari scoring system,[11] based on duration of diarrhea, maximum number of stools passed per day, vomiting, grade of fever, severity of dehydration, altered sensorium and requirement of intravenous fluids. Since, accurate temperature measurements were not possible in the field, it was recorded as normal, low-grade or high-grade fever in the history, as reported by the caregivers. An episode was considered mild for a score of ≤ 5, moderately severe for a score of >5 to 10, and severe for a score of >10. Case of severe dehydration got hospitalized and managed.

Collection of Samples

All stool samples collected in the hospitals were transported within 2 hours to the testing laboratory. Detection of VP6 antigen in diarrheal stool sample was carried out by using 'IVD Research Inc.' Rotavirus antigen was detected by using ELISA. Out of 110 samples, 34 (30.9%) were positive for VP6 antigen suggestive of Rotavirus-A.

RNA PAGE (Electropherotyping)

Rotavirus double-stranded RNA was extracted from stool of infected stool samples by using Trizol-LS reagent (Life Technologies, Rockville, Md). All fecal specimens were analyzed by polyacrylamide gel electrophoresis (PAGE) to confirm Rotavirus group A and for presence of group B or C rotavirus double-stranded RNA (dsRNA), if any. The methods have been described in detail elsewhere.[12]

RNA Extraction using QIAamp Viral RNA Mini Kit

Phosphate buffer saline suspensions of fecal samples 10% were clarified by centrifugation at 10,000 gm for 10 minutes. Genomic RNA was extracted from 140 µl of 10% stool suspensions using a spin column technique according to the manufacturer's instructions. (QIAamp Viral RNA mini kit from QIAGEN GmbH, Hilden, Germany).

Reverse Transcription-PCR (RT-PCR) Genotyping

In view of high cost involved in genotyping, stool samples, found positive for rotavirus either by ELISA or by electropherotyping were subjected to genotyping by RT PCR. Prior standardization was done for amplification of Rotavirus RNA, identifying the specific G and P genotypes. All the RT-PCRs were performed with viral RNA extracted from reference samples as the positive controls and water as the negative control.

Rotavirus G Genotyping

Reverse transcription was used to synthesize the cDNA corresponding to the genomic segment encoding VP7, and the characterization of G genotypes was performed with specific oligonucleotide primers according to a previously described system.[13,14] The cocktail of primers used for typing was the common primer 9con1 and the G type-specific primers G1 (9T1-1), G2 (9T1-2), G3 (9T-3P), G4 (9T-4) and G9 (9T-9) and G8 (MW-8). The sizes of the G type-specific PCR products were 110 bp (G9), 160 bp (G1), 246 bp (G2), 466 bp (G4) and 405 bp (G3). The reaction was carried out with an initial reverse transcription step at 45°C for 1 hour, followed by 40 cycles of amplification (30 sec at 94°C, 45 sec at 45°C, 1 min at 72°C), and a final extension of 10 min at 72°C in a thermal cycler (peqLAB).

Rotavirus P Genotyping

A semi nested multiplex type specific PCR was used for P typing. In the first round of PCR, consensus primers Con3 and Con2 (Complementary to conserved region of VP4 gene) were used to amplify an 877 bp region. The consensus Con3 and the P type specific primers for P4 (2T-1), P6 (3T-1), P8 (1T-1)[13] were used during the second round of PCR. The PCR mixture composition and thermal conditions for the first and second rounds of amplification were the same for the G typing, except for the primers used for amplification.

Agarose Gel Electrophoresis

All amplified PCR products after the first and second rounds of PCR were subjected to electrophoresis on 2% agarose gel containing 0.5 mg/ml of ethidium bromide and observed under ultraviolet light. Specific segment sizes for different G and P genotypes were observed.

Statistical Methods

Sample size was calculated using the incidence rate in the community (20%), with a power 80% and alpha error of 0.05. Data was analyzed using Students t-test and Chi-square test. Virus phenotypes and genotypes were compared with their respective disease severity in terms of vasikari scores on clinical parameters recorded on pretested proforma for each case.

RESULTS

110 children between 6 and 60 months (M 68, F 42) with suspected cases of acute viral diarrhea were screened by ELISA after excluding invasive (Bacterial) diarrhea by history of presentation and stool examination. 34 (30.9%) of them came positive for Rotavirus group.

Age, Seasonal Predilection and Duration

58.8% of Rotavirus positive cases were in the age group of 6 to 12 months, 23.5% in 13 to 24 months and 14.7% in 25 to 60 months. Rotavirus diarrhea was seen round the year but 67.6% occurred during colder months (November-February), 23.5% during March-May and 8.8% during July-October in this study. In majority of Rotavirus positive cases, 2.9% lasted only for 1 day, 8.8% for 2 days, 17.6% for 3 days, 47% for 4 days and 20.53% for 5 days.

Disease Severity

Of 34 cases of diarrhea positive for Rotavirus A on ELISA, 14.7% were having no dehydration, 38.2% had mild, 35.2% moderate and 11.7% severe dehydration. Among children whose stool samples were negative for Rotavirus, 47.36% had no dehydration, 22.36% had mild, 18.42 moderate and 11.8% severe dehydration. Loose, watery motion was the most common complaint (85.2%) followed by vomiting (61.76%) among Rotavirus positive cases. Fever was seen in 35.29% and respiratory symptoms in 8.82% of rotavirus positive cases. In rotavirus negative cases, watery stool was in 78.9% cases, vomiting in 34.2%, fever in 30.26% and respiratory symptoms in 3.9% cases.

RNA PAGE Electropherotyping

Out of 34 samples, 32 showed typical Rota-A type bands. Rest 2 showed Rota-B type bands in addition, suggesting mixed infection. None of ELISA negative samples were found positive either for Rotavirus-A or B in electropherotyping, demonstrating its reliability at par ELISA.

Long and Short Electropherotypes (Fig. 1)

Of 39 positive for purely Rotavirus-A, 17 were found to be of Long-electropherotype (Long-E) type (53.1%) and the rest were of short-electropherotype (Short-E) types (46.9%). The association of electropherosubtypes with clinical disease severity was assessed. Long-E types were found to be associated with more severe disease. Eleven, i.e. 64.6% of all Long E type caused moderate and severe dehydration as compared to only 2, i.e. 13.3% of all Short E type causing moderate dehydration only, but no severe dehydration. This was highly significant ($p < 0.009$).

Genotyping

Out of 34 stool samples positive for Rotavirus, 15 were subjected to RT-PCR by computer generated randomization. Three different G types were detected: G1, G4 and G9. Of these, G9 was the most prevalent genotype (26.66% alone and 6.6% mixed with G1), followed by G1 and G4 (13.3% each). Rests were untypable (40%) by the set of primers used. Nine could be genotyped for VP4 and 4 could be genotyped for VP7. The P types found were P(4) 20% and P(6) 6.7%. Others were untypable. The only G-P combinations seen was G1P(4) 26%. Although G9 was the commonest genotype in our study, its P type could not be typed in most. There was only one fully genotyped strain of G1P (4) in our study (Table 1).

DISCUSSION

Age, Seasonal Predilection and Duration

In the US and Europe, Rotavirus infection occurs primarily during the winter. Some studies from India suggested the disease occurs year-round there. The peak of infection occurs during the winter[16] while another study found 2 peaks per year.[17] In two other Indian studies, one from north and the other from south (Kerala), no seasonal pattern was found.[17,18] The temporal distribution of Rotavirus incidence was observed in this study throughout the year, with higher incidence during colder season (November-February).

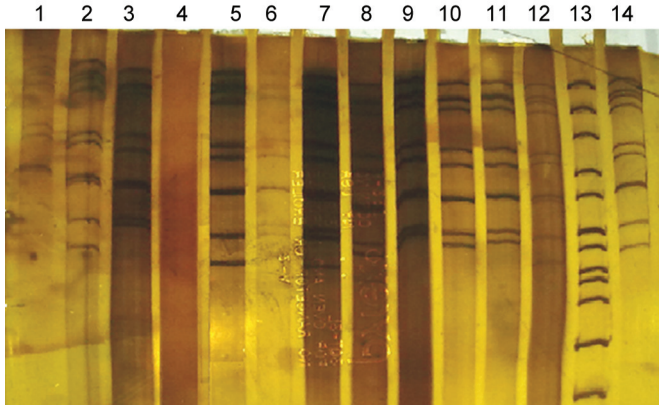

Fig. 1: PAGE of Rotaviruses showing the long and short electropherotyping [Lane 3, 6, 9, 10-13: long type; Lane 1, 2, 5, 12 and 14: shorter type; Lane 7, 8: untypable (mixed infection, also mimicking Rotavirus-B type)]

Table 1: G(P) genotypes of Rotavirus in fecal samples from symptomatic infants and children

No. of patients	*ELISA*	*Electro-pherotyping*	*G-Genotype*	*P-Genotype*
1	Positive	Long	G1 + G9	Untypable
2	Positive	Long	Untypable	P4
3	Positive	Long	G1	P4
4	Positive	Short	G9	Untypable
5	Positive	Short	G1	Untypable
6	Positive	Short	G4	Negative
7	Positive	Short	G9	Untypable
8	Positive	Short	G4	Negative
9	Positive	Short	Untypable	P4
10	Positive	Long	G9	Negative
11	Positive	Short	G9	Untypable
12	Positive	Long	Untypable	P6
13	Positive	Long	Untypable	Untypable
14	Positive	Long	Untypable	Untypable*
15	Positive	Long	Untypable	Untypable*

*An unusual pattern in electropherotyping

Incidence

In this study, Rotavirus was found positive in approximately 31% of clinically suspected viral diarrhea which compares well with findings of Mathew et al from Kerala (35.8%),[18] Kelkar et al from Pune (28-30%)[19] and in studies reported from Delhi (33.33%).[20] The incidence at Navi Mumbai in this study seems to be much higher than reported from other centers in India such as Chandigarh (19%), Vellore (18%) and Chennai[21] (22%) and less as compared to studies from Manipur (41%).

Age

In the present study, the highest (58.8%) incidence of Rotavirus diarrhea was seen among 6 to 12 months of the age which is comparable to reports from India and abroad. In our study, it was least common during late infancy which might be due to continued protection of maternal antibodies transferred transplacentally as well as from breastfeeding.

Seasonal Predilection

In this study, (67.6%) cases occurred during colder months November to February, 23.5% during March to May and 8.8% during July to October. Our results correlate well with the study of Kelkar et al (Pune, about 200 km from our location), who got 61.8% positive cases in winter and 9.8% in rainy season.

Symptomatology

Fever, vomiting and respiratory symptoms were present in 35.29, 61.76 and 8.82% respectively of all children having diarrhea whose stool samples were positive for Rotavirus in our study. Shariff et al[22] in their study recorded fever in 26% cases. In our study, vomiting was present in 61.76% of cases corresponding well with Sheriff et al (56%) but is lower than reported by Nafi et al (86.4%). Mathew et al from Kerala[18] recently reported mild fever in 32.8%, moderate in 37.1% and >38.7°C in 46.7% of all fever cases.

Degree of Dehydration

Of Rotavirus positive diarrhea cases in our study, 14.7% had no dehydration. Mild dehydration was present in 38.2%, moderate in 35.2% and severe dehydration in 11.7% of cases. Our results are slightly higher than Shariff et al and Nafi et al who recorded dehydration in 76% cases and 72.3% cases respectively on admission.[22,23] In their study only 11 (7.4%) presented with severe dehydration, while the remaining 96 (64.9%) were considered to have mild dehydration. Patients with dehydration in the current study were significantly younger than those without dehydration. Among the non-Rotavirus diarrhea cases, 46.36% had no dehydration, 22.36% had mild dehydration, 14.42% had moderate dehydration and 11.8% had severe dehydration; just similar to Rotavirus positive cases of diarrhea. This drove away the myth that Rotavirus diarrhea is often associated with severe dehydration while other forms were not. However, Mathew et al[18] studying Rotavirus positive hospitalized children only reported no dehydration in 32.2%, some dehydration in 45.3% and severe dehydration in 44.4% of cases. This could be a sample bias as their study was conducted on hospitalized children who were obviously admitted for treatment of severe diarrhea, needing parenteral fluid therapy.

ELISA and Electropherotyping

In our study, out of the 32 samples which were positive for Rotavirus by PAGE (Electropherotyping), 53.1% showed long electropherotypes and 46.9% showed short electropherotypes (*see* Fig. 1). A study from Vellore[24] found long type predominance over shorter type. Out of 117 samples, 75 (64.1%) showed long patterns and 25 (21%) short patterns. Das et al[25] also found a predominance of long electropherotypes. In contrast to our findings, the study of from Chennai[26] found a predominance of Short E types as compared to Long E types. This variation needs to be elucidated further by larger studies. A strong association of Short and Long E types with subgroups I and II respectively have been observed by some workers. Studies have correlated the presence of severe gastroenteritis and found subgroup II to be associated with more severe disease. In our study, the Long E types were associated with more severe illness in majority of the cases as compared to the Short E types. 64.63% cases of Long E type had severe disease (moderate to severe dehydration) in

contrast to 13.36% of Short E type causing severe disease. The difference was highly significant (p = 0.009) not reported so far.

Genotyping

Out of 15 ELISA and electropherotype positive samples randomized from 34 by taking alternate samples, RT PCR failed to detect any G-type in 4 samples and no P-type in 9 samples. This could be due to numerous factors including small sample volume picked up for analysis due to cost constraints, time of sampling, inhibitors in the samples that could have masked PCR reaction and storage time before analysis.

Out of 15 samples tested, 5 were positive for G9 (33.33%). Such higher prevalence of G9 not been reported from any part of India earlier except Kerala[18] although larger sample size required to confirm this. One sample positive for Rotavirus genotype G9, also exhibited G1 genotype, suggesting ongoing genetic shift between 2 different genotypes undergoing genetic reshuffle while infecting the same host cell. A study conducted in Kerala in the corresponding period[18] had also shown higher G9 genotypes (29%) with G1 P(8) 49.7%, G9 P(8) 26.4%, G2 P(4) 5.5%, G9 P(4) 2.6%, G12 P(6) 1.3%, G1 P(6) 0.8%, G12 P(8) 0.8%, G1 P(4) 0.2%, G1 P(Untypable) 0.2%, G9 P(Untypable) 2.4% and others, mixed to be 9.2%.

All recent studies confirm the diversity of Rotavirus strains much greater than previously recognized as their epidemiology is changing rapidly. Specific genotypes, such as G9 and G12, are emerging in various parts of the world, particularly in developing countries where G1 or G2 were the only genotypes earlier. In a review article by Broor et al[27] on molecular epidemiology of Rotaviruses in India, much genetic and antigenic diversity was highlighted. Electropherotyping demonstrated multiple electropherotypes co-circulating at a given time in a particular community, leading to extensive genomic variation and appearance of new strains**.**

In India, the most common G types are G1 and G2 and P types were of P(4) and P(8). Of late, G9 is being reported in isolated manner as an emerging strain besides P(6) strains of Bovine population. Our study has shown predominance of G9 strain (33.33% of positive samples) followed by G4 and G1. One sample was positive for P(6) strain. One strain had multiple G types. Though G9 was the commonest genotype in our study, its P type could not be detected in any. There was only one fully genotyped strain, G1 P(4) type. The findings are suggestive of re-emergence of diverse strain in the region. Besides pointing toward wide variation of Rotavirus serotypes, the present study raised the possibility of several novel strains circulating in Navi Mumbai region, including the emerging G9 and P6 types. Larger sample size needs to be studied before drawing important conclusions for the region.

The diversity of Rotavirus strains and its high incidence emphasize that vaccines need to be redesigned against a broad range of strains. Currently one monovalent (G1) vaccine (Rotarix, Glaxo) and one polyvalent human-bovine reassortant with G1, 2, 3 4 serotypes (Rotateq, MSD) oral attenuated vaccines are available. These vaccines, which were earlier showing protective efficacy of over 85% in European and American population with G1-3 strains in the eighties, recently showed a mere 35 to 46% efficacy in the Middle East, South Africa, Malawi and other low-income countries; where G9 and G12 had emerged as predominant strains.[28,29] The manufacturers still drive these vaccines with claim of 'Cross protection' and 'Herd immunity' on basis of few observational studies, but not without conflict of interests. Such claims are strictly not consistent with scientific concept of cross protection in viral infections. No prospective seroconversion study is available from our country for confirmation of such claims.

With G9 and other non-G1, G2, G3 serotypes now emerging in certain parts of our country as was seen in the Middle East, Latin America and Africa, similar antigenic shifts are certainly a possibility in other parts of the country as well by mutation, genetic shifts, drifts, due to fast population dynamics and climate change. Larger Multicentric, double-blind and prospective studies are desirable, besides looking for role of other enteric viruses responsible for causation of diarrhea in the Rotavirus negative samples. Our population generally behaved differently to oral vaccines (e.g. OPV) with heavily loaded gut-microbiota as well as high parasite load as compared to the Westerners. Moreover, individual variation in susceptibility to viral infections needs to be taken into consideration, than blindly banking upon mass scale vaccination at exorbitant cost. In outbreak studies of a GII-3 and a GII-4 Norovirus strain, association between HBGA phenotypes and viral infection was established.[30] Such hypothesis was extrapolated to show human susceptibility and resistance to Norwalk virus infection.[31] Persons carrying more than one functional FUT2 allele, expressing al2 fucosyl-transferase2, were termed as secretors and can express the A and B blood group antigens as well as H-type 1 and Lewis b (Leb) antigens on mucosa as well as secretions.[34] Same needs to be explored in case of Rotavirus and other enteric viruses which have not been studied so far.

The newer indigenous vaccine from India, using bovine re-assortant neonatal strain 116E, of G9P(11) type, initially detected at the AIIMS, New Delhi, was developed by the

department of Biotechnology (Government of India), cleared by the US FDA and under manufacture by Bharat Biotech is scheduled for a phase-IV study. Promised at a cost of mere 1$ per dose, has shown a slightly better efficacy of 53.6% (95% CI 35.0-66.9; $p < 0.001$) against severe Rotavirus acute gastroenteritis and good tolerability in Phase-III study.[32,33] There is a genuine need to develop cheaper but effective vaccine selectively against locally prevalent strains in the region, revised from time to time, as in case of Influenza. May be, an injectable form that could be integrated with other UIP vaccines is a convenient option for the resource scarce countries.

SUMMARY AND CONCLUSION

Rotavirus affected children mainly below 2 years (82.3%) in the suburban region of Navi Mumbai, India. The highest (58.8%) incidence was seen in 6 to 12 months of the age. Peak incidence was found in December (19.05%), followed by January (17.8%). Maximum Rotavirus positive samples (67.6%) were isolated in winter months. Average duration of Rotavirus diarrhea was 3 to 5 days, 80% resolved within 4 days. Predominant associated symptoms in Rotavirus diarrhea were vomiting (61.76%), fever (35.29%) and respiratory symptoms (8.82%), in that order. Mild dehydration was present in 38.2%, moderate in 35.2% and severe in 11.7% of cases of confirmed rotavirus diarrhea in this study. Of 110 cases of diarrhea in the age group of 6 months to 5 years, 34 (30.90%) were positive for Rotavirus-A. Of them, 2 were of mixed A and B serotypes.

Of 29 Rota-A confirmed by electropherotype, 53.1% were of long type and 46.9% short electropherotype. Long ones were associated with more severe dehydration as compared to short ones. The difference was highly significant ($p < 0.009$). G9 was found to be most prevalent genotype (33.33%), followed by G1 (20%) and G4 (13.3%). It suggested emergence of diverse strains in the region. One was positive both for G1 as well as G9, showing antigenic shift by ongoing genetic shuffle between 2 genotypes, infecting the same host cell. Of P types identified, P(4) were 20%, P(6) 6.7% and rest were untypable. The only G-P combinations seen was G1P(4) (26%). There is a genuine need to develop cheaper, effective and indigenous but effective vaccines, directed against the country specific strains. With the right policy prioritization at national level, this will not remain as a distant dream to include it in the national immunization program free, delivering it at grass-root levels, where it is needed the most.

REFERENCES

1. CDC and prevention of Rotavirus surveillance world-wide, 2001-2008. MMWR Morbidity and mortality weekly report 2008;57: 1255-1257.
2. Kosek M, Bern C, Guerrant RL. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull World Health Organ 2003;81:197-204.
3. Bern C, Martines J, de Zoysa I, Glass RI. The magnitude of the global problem of diarrhoeal disease: a 10-year update. Bull World Health Organ 1972;70:705-714.
4. Parashar UD, Bresee JS, Gentch JR, Glass RI. Rotavirus. Emerg Infect Dis 1998;4:561-570.
5. Glass RI, Bresee JS, Parashar U, Miller M, Gentsch JR. Rotavirus vaccines at the threshold. Nat Med 1997;3:1324-1325.
6. WHO scientific working group, Rotavirus and other viral diarrhoeas Bulletin of the World Health Organization, 58 1980; (2):183-198.
7. Jain V, Parashar UD, Glass RI, Bhan MK. Epidemiology of Rotavirus in India. Ind J Pediatr 2001;68:855-862.
8. Parashar UD, Gibson CJ, Bresse JS, Glass RI. Rotavirus and severe childhood diarrhea. Emerg Infect Dis 2006;12:304-306.
9. Hodges K, Gill R. Infectious diarrhea: cellular and molecular mechanisms. Gut Microbes 2010 January/February;1(1):4-21.
10. Chandran A, Fitzwater S, Zhen A, Santosham M. Prevention of Rotavirus gastroenteritis in infants and children; rotavirus vaccine safety, efficacy and potential impact. Biologics 2010;4:213-229.
11. Ruuska T, Vesihkari T. Rotavirus disease in finnish children: use of numerical scores for clinical severity of diarrhoeal episodes 1990;22(3):259-267.
12. Albert MJ, Bishop RF, Shann FA. Epidemiology of rotavirus diarrhea in Highlands of Papua NewGuinea 1979, as revealed by electrophoresis of genome RNA. J Clin Microbiol 1983;17(1):162-164.
13. Das BK, Gentsch JR, Cicirello HG, Woods PA, Gupta A, Ramachandran M, Kumar R, Bhan MK, Glass RI. Characterization of Rotavirus strains from newborns in New Delhi, India. J Clin Microbiol 1994;32:1820-1822.
14. Gentsch JR, Woods PA, Ramachandran M, Das BK, Leite JP, Alfieri A, Kumar R, Bhan MK, Glass RI. Review of G and P typing results from a global collection of rotavirus strains: implications for vaccine development. J Infect Dis 1996;174:S30-S36.
15. Chakravarti A, Kumar S, Mittal SK, Broor S. Clinical and epidemiological features of acute gastroenteritis caused by human Rotavirus subgroups. J Diarrhoeal Dis Res1992;10:21-24.
16. Singh V, Broor S, Mehta S, Mehta SK. Clinical, epidemiological features of acute gastroenteritis Assoc with human rotavirus subgroup 1 and 2 in North India. Indian J Gastroenterol 1989; 8:23-25.
17. Yachha SK, Singh V, Kanwar SS, Mehta S. Epidemiology, subgroups and serotypes of rotavirus diarrhea in north Indian communities. Indian Pediatr 1994;31:27-33.
18. Mathew MA, Paulose A, Chitralekha A, Nair MKC, Kang G, Kilgore P. Prevalence of Rotavirus diarrhea among Hospitalised Under-5 children. Indian Pediatrics 2014;51:27-31.
19. Borade A, Bais AS, Bapat V, Dhongade R. Characteristics of Rotavirus gastroenteritis in hospitalized children in Pune. Indian J Med Sc 2010;64(5):210-218.
20. Ramani S, Kang G. Burden of disease and molecular epidemiology of group A Rotavirus infections in India. Indian J Med Res 2007 May;125:619-632.
21. Kang G, Kelkar SD, Chitambar SD, Ray P, Naik T. Epidemiological profile of Rotaviral infection in India: challenges for the 21st century. J Infect Dis 2005 Sep 1;192(Suppl 1):S120-S126.
22. Shariff M, Deb M, Singh R. A study of diarrhoea among children in eastern Nepal with special reference to Rotavirus. Ind J Med Microbiol 2003 Apr-Jun;21(2):87-90.
23. Nafi O. Rotavirus gastroenteritis among children aged under 5 years in Al Karak, Jordan. East Mediterr Health J 2009;16(10):1064-1069.

24. Brown DW, Mathan MM, Mathew M, Martin R, Beards GM, Mathan VI. Rotavirus epidemiology in Vellore, South India: group, subgroup, serotype and electropherotype. J Clin Microbiol 1988 Nov;26(11):2410-2414.
25. Dash SK, Kumar K, Tewari A, Varshney P, Goel A, Bhatia AK. Detection of Rotavirus from hospitalized diarrhea children in UP, India. Indian J Microbiol 2012 July-Sept;52(3):472-477.
26. Saravanan M, Parthiban M, Ramadass P. Genotyping of Rotavirus of neonatal calves by nested-multiplex PCR. Veterinarski Arhiv 2006;76(6):497-505.
27. Broor S, Ghosh D, Mathur P. Molecular epidemiology of Rota viruses in India. Indian J Med Res 2003 Aug;118:59-67.
28. Cunliffe NA, Gondwe JS, Graham SM, Thindwa BD, Dove W, Broadhead RL, Molyneux ME, Hart CA. Rotavirus strain diversity in Blantrye, Malawi, 1997-99. J Clin Microbiol 2001;39: 836-843.
29. Madhi SA, Cunlife NA, Steel D, et al. Effect of human Rotavirus vaccine on severe diarrhea in African infants. NEJM 2010;362: 289-298.
30. Miao Jin MT, Xie H, Duan Z, Jiang X, Fang Z. Outbreak studies of a GII-3 and a GII-4 norovirus revealed an association between HBGA phenotypes and viral infection. J Med Virol 2008 July;80(7): 1296-1301.
31. Lindesmith L, Moe C, Marionneau S, et al. Human susceptibility and resistance to Norwalk virus infection. Nat Med 2003;9: 548-553.
32. Bhandari N, Sharma P, Taneja S, Kumar T, Chandola TR, et al. A dose-escalation safety and immunogenicity study of live attenuated oral Rotavirus vaccine 116E in infants: a randomized, double blind placebo-control trial. J Infectious Dis 2009;200: 421-429.
33. Bhandari N, Rongsen-Chandola T, Bavdekar A, John J, et al. Efficacy of monovalent human-bovine (116E) rotavirus vaccine in Indian infants: double blind, placebo controlled trial. www. thelanct.com. 2014 March 12;1-8.
34. Nordgren J, Le´on W. Nitiema, Ouermi D, Simpore J, Svensson L. Host genetic factors affect susceptibility to norovirus infections in burkina faso. Plos One 2013;8(7):e69557.

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ORIGINAL ARTICLE

Peripheral Pulse Morphology for Early Detection of Coronary Artery Disease

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ABSTRACT

Based on the past observation of recording abnormal impedance cardiogram (ICG) in 13% normal subjects and subsequent detection of coronary artery disease (CAD) in majority of these cases, led the authors to record peripheral impedance plethysmograms (IPG) in control subjects and patients using impedance cardiovasograph, developed by electronics division, Bhabha Atomic Research Centre (BARC). Analysis of peripheral plethysmograms, thus recorded, has shown 8 dominant morphological patterns of the peripheral pulses depending upon their status of health. In cognizance of these observations, different methods of pattern analysis were used for pattern identification. Fourier Transform based method has been observed to yield higher diagnostic yield. Morphology index (MI) of the peripheral pulse derived from this method was observed to vary from 0.28 to 1, the former indicating the poorest and later the normal health. Among 100 subjects suffering from various disorders, 8 patients with coronary artery disease have recorded average index to be between 0.30 and 0.45.

Keywords: Impedance plethysmograph, Coronary artery disease, Peripheral pulse analysis, Peripheral pulse morphology, Morphology index.

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INTRODUCTION

Bhabha Atomic Research Centre (BARC) developed an impedance plethysmograph (IPG) in 1978 and installed at Department of Surgery, Seth GS Medical College and KEM Hospital and Department of Medicine, Grant Medical College and JJ Hospital, Mumbai for the assessment of central and peripheral blood flow in the human body. Extensive clinical trials on thousands of normal subjects and patients established the sensitivity and specificity of this indigenously developed technique to be 96 and 98% for the diagnosis of peripheral arterial occlusive disease.[1] Deshpande et al[2] have shown different morphological patterns (Fig. 1) in a group of 103 subjects, without any demonstrable cardiovascular disorder termed, as type A, type B, type C and type D waveforms. Type D waveform recorded in 13 subjects is similar to those recorded in patients with tricuspid regurgitation and those with myocardial infarction. In the absence of clinical correlation, these cases have been regarded as false positives. However, majority of them suffered heart attack during subsequent 15 years. This suggested predictive diagnostic potential of this pattern.

BARC's instrument has undergone several renovations during the past 31 years such as microprocessor based impedance plethysmograph, introduction of simple and reliable calibration for dZ/dt waveform,[3] Correction of formula for estimation of peripheral blood flow,[4] introduction of normalized dZ/dt waveform for easy assessment of peripheral blood flow,[5] PC based impedance cardiovasograph system[6] and variability analyzer.[7] These instruments have been used by medical fraternity for different clinical applications. Arya Vaidya Sala (Kotakkal) and Ayurvedic Hitaishini Trust (Thane) have specifically used the variability analyzer for recording the peripheral pulse and studying the variability in heart rate and peripheral blood flow.[8,9]

While analyzing variability analyzer data, it was noticed that the morphology of the peripheral pulse varied as a function of time in a given individual and also from individual to individual. It was observed that in a span of 300 seconds, an individual has a dominant pattern most of the time with other patterns interposing intermittently. A closer examination of the data in all the 300 subjects classified these pulse patterns in 8 different morphologies as shown in Figure 2. Top left is the pulse morphology, commonly observed in normal subjects and bottom right is the pulse morphology, commonly observed in patients with severe coronary artery disease.

Karamchandani et al[10,11] have tried several methods for automatic identification of these patterns including dynamic time warping, parallel support vector, etc. Classification of these waveforms using dynamic time warping yielded an accuracy of more than 94%, efficient predictive values, and statistics evaluating measures such as MCC and kappa

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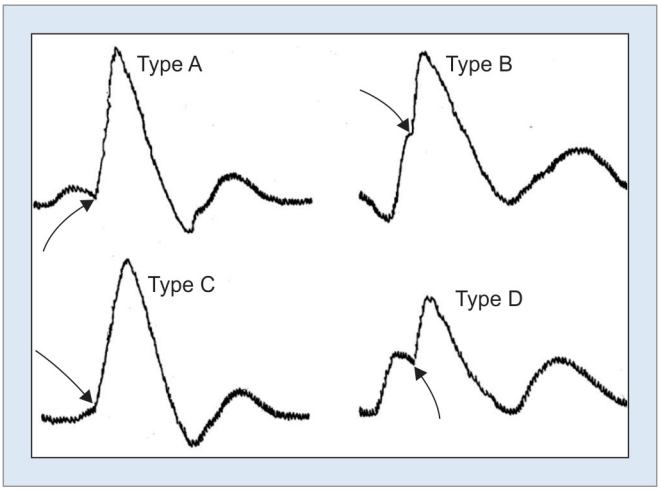


Fig. 1: Variation in the morphology of ICG waveforms in subjects without any demonstrable cardiovascular disorder. B-point, indicated by the arrow, is clearly discernible in type A and type C waveforms recorded from 73.8% of the presumably normal subjects. However, 26.2% of the subjects have recorded waveforms with morphologies similar to that of type B or type D. The waveforms of latter types invariably caused inaccuracy in the computation of hemodynamic parameters

coefficients. Using data-mining technique, such as parallel support vector machine, they have developed an online technique as an aid to the physician for pattern recognition. The accuracy of the SVM model is largely dependent on the selection of the kernel parameters such as C and g, which are obtained using cross-validation technique. They have obtained a clinical correlation of over 85% with cardiovascular conditions such as myocardial infarction, cirrhosis of liver and pulmonary tuberculosis.

These efforts helped in identification of pulse patterns; however they were inadequate for the study of morphology variability. In order to assign a numerical value to pulse pattern, named as morphology index (MI), K-factor and Fisher's ratio have been used by others in the past and have met partial success. We have used short term fast fourier transform (FFT) for the same purpose. The method and results are presented in this paper.

METHODS

Peripheral pulse analyzer, developed at Electronics Division BARC, has been used for this investigation. It comprises a sine-wave oscillator, voltage to current converter, three sensing amplifiers along with analog processing circuits, a low power microcontroller and a bluetooth controller communicating with a personal computer as shown in Figure 3. These signals are acquired at a rate of 500 samples per second and communicated to personal computer through Bluetooth controller for further processing and analysis.

The firmware includes acquisition of all the user selected signals at selectable rate and sending to PC through bluetooth controller. The application software has two parts; acquisition and processing. During acquisition, after entering the personal data and basic settings for the subject, click on AQUIRE button starts data acquisition till the same button is re-clicked or 275 seconds have elapsed, whichever is earlier. At the end of acquisition, the data is saved in the prescribed file format. Also, the file can be converted to ASCII format and saved for processing on other software packages. For processing the file, the patient data is loaded by clicking on LOAD, signals are selected for processing and Selection Panel is clicked. Cursor is placed on third systolic peak in dZ3 (dZ3/dt is abbreviated as dZ3) and LOCATE PEAK is clicked. This automatically highlights all the systolic peaks in the signal.

Since data of one cardiac cycle gives poor resolution due to limited number of samples, 511 samples on the left side and 512 samples on the right side of the peak are given as input for short-term FFT for higher resolution as shown in Flow Chart 1. FFT of these 1024 data samples is computed

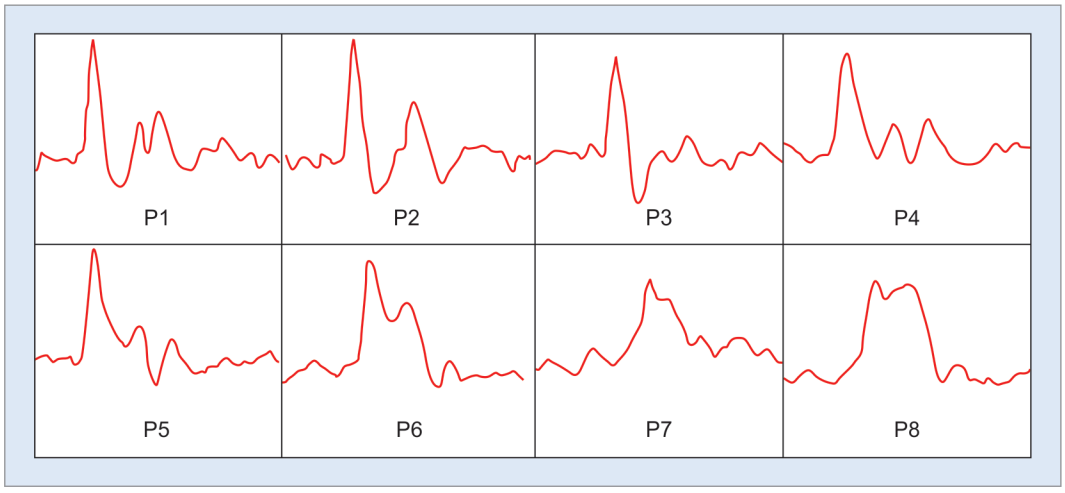


Fig. 2: Eight different morphological patterns of peripheral pulse

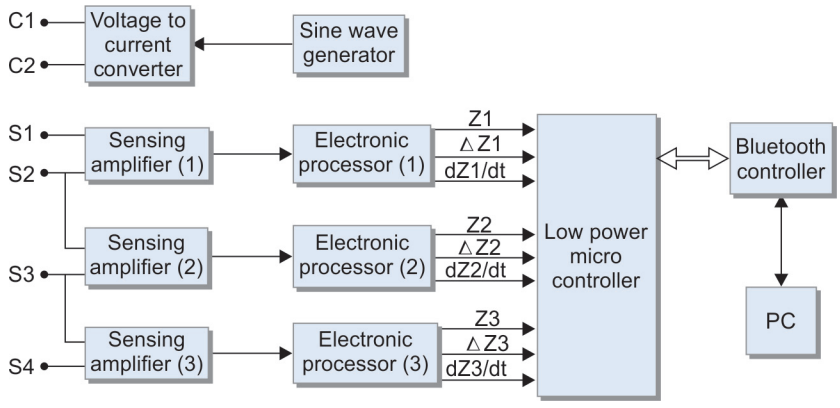


Fig. 3: The schematic diagram of the peripheral pulse analyzer developed by electronics division BARC

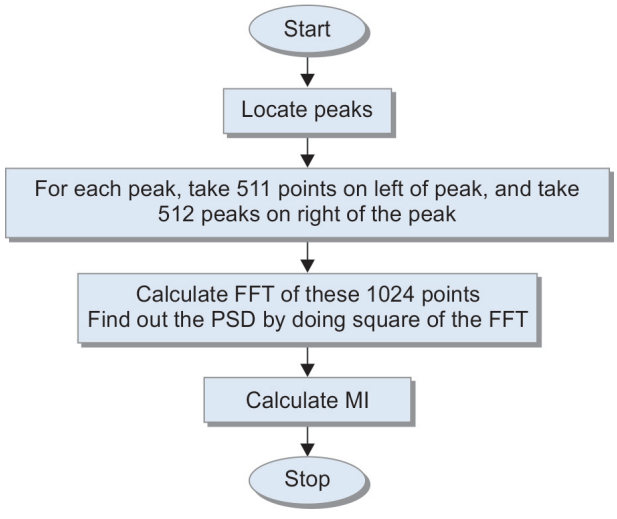
and power spectral density (PSD) is obtained. Hanning window is used for obtaining smooth FFT. The MI is then computed from the FFT data using following formula:

$$MI = \frac{\sum_{i=6}^{i=127} PSD\ (i)}{\sum_{i=2}^{i=127} PSD\ (i)}$$

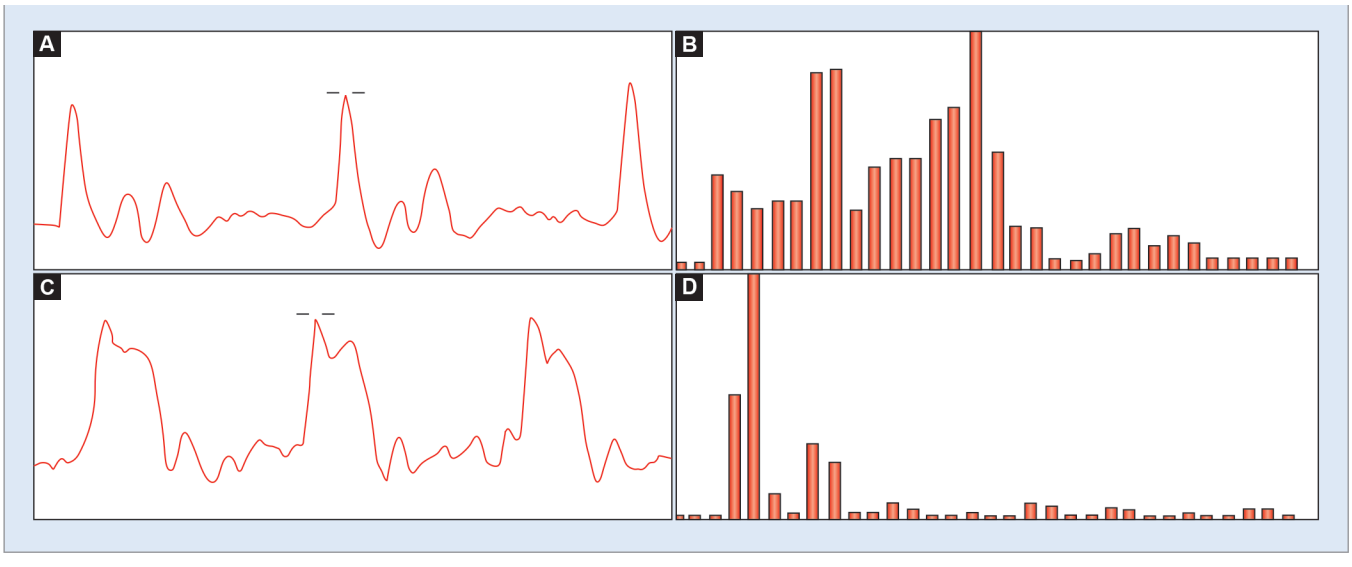
where, PSD (i) is the sum of squares of real and imaginary Fourier coefficient as obtained from FFT. The first two coefficient are ignored as they have high values due to DC component.

Figures 4A to D show the output of this algorithm in terms of peripheral pulses and their short-term FFT. Only 32 coefficients are shown for the purpose of clarity. For morphology pattern 1, high frequency components are dominant with the result the MI is closer to unity. Whereas, for morphology pattern 8, the lower frequency components are dominant with the result that MI is approaching toward zero. The other patterns have values ranging between 0.3 and 1.

Flow Chart 1: Calculation of MI



This algorithm has been incorporated in the application software of the instrument for obtaining the morphology variability as described below.



Figs 4A to D: The peripheral pulse recorded from a normal subject (A) and a patient with severe coronary artery disease (C). The short term FFT is given in (B) and (D) respectively (*Courtesy*: Jindal et al)

RESULTS

Figure 5 shows the selection panel of the system. dZ1, dZ2 and dZ3 represent the peripheral pulses at three locations in wrist segment in a subject. HRdZ3 shows the heart rate variability in time domain. Three graphs on the bottom left give the blood flow variability and those on bottom middle give the morphology variability.

As can be seen from the figure MI_dZ3 shows wide variation in the morphology of the pulse ranging from 0.30 to 0.89. The short-term FFT shown in the graph by the side of HR_dZ3 graph is for the peripheral pulse for MI equal to 0.89. The corresponding pulse pattern can be seen in the graph labelled dZ3, which resembles pattern P1.

The instrument has been used for screening nearly 100 subjects suffering from various disorders. Eight patients suffering from coronary heart disease have recorded patterns P6 to P8, with average morphology index ranging from 0.3 to 0.45.

DISCUSSION

Different morphology of the impedance cardiogram has been observed in control subjects[2] and patients[12] in the past several decades. Change in morphology in diseased state is understandable but the same cannot be explained in control subjects. Analysis of vector impedance cardiogram[13] in control subjects has shown that border line hypertensive or smokers record ICG parameters outside the range of control values. These observations led the authors to have a follow-up on 13 subjects recording type D waveform. 11 out of 13 cases suffered heart attack by the year 2000. This observation suggested the importance of morphological changes in control subjects.

Physiological variability is one of the recent investigations added during the last two decades for the objective assessment of autonomic function and for the assessment of prognosis in severe sickness like myocardial infarction, diabetic neuropathy, etc.[14] In addition to heart rate variability studied worldwide, few researchers have studied blood pressure variability and peripheral blood flow variability.[7-9] Changes in the morphology of the peripheral pulse were noticed during these studies and different methods were tried to quantify the morphological changes[10,11] with a yield of 80 to 90%.

Authors have extended the work of earlier researchers on morphological variations. The extended work is confined to Fourier analysis, which is well understood and extensively used for the past two centuries. In fact tomographic imaging has also become a reality due to Fourier analysis. Using Fourier Transform the authors have derived a new index called MI, which gives the ratio of high frequency spectral components to the total spectral density. In other words, it is a composite measure of distensibility, elasticity and compliance of arteries.

As observed during the study, the MI observed in 100 subjects varies from 0.3 to 0.89. The highest values have been recorded in asymptomatic volunteers and lowest

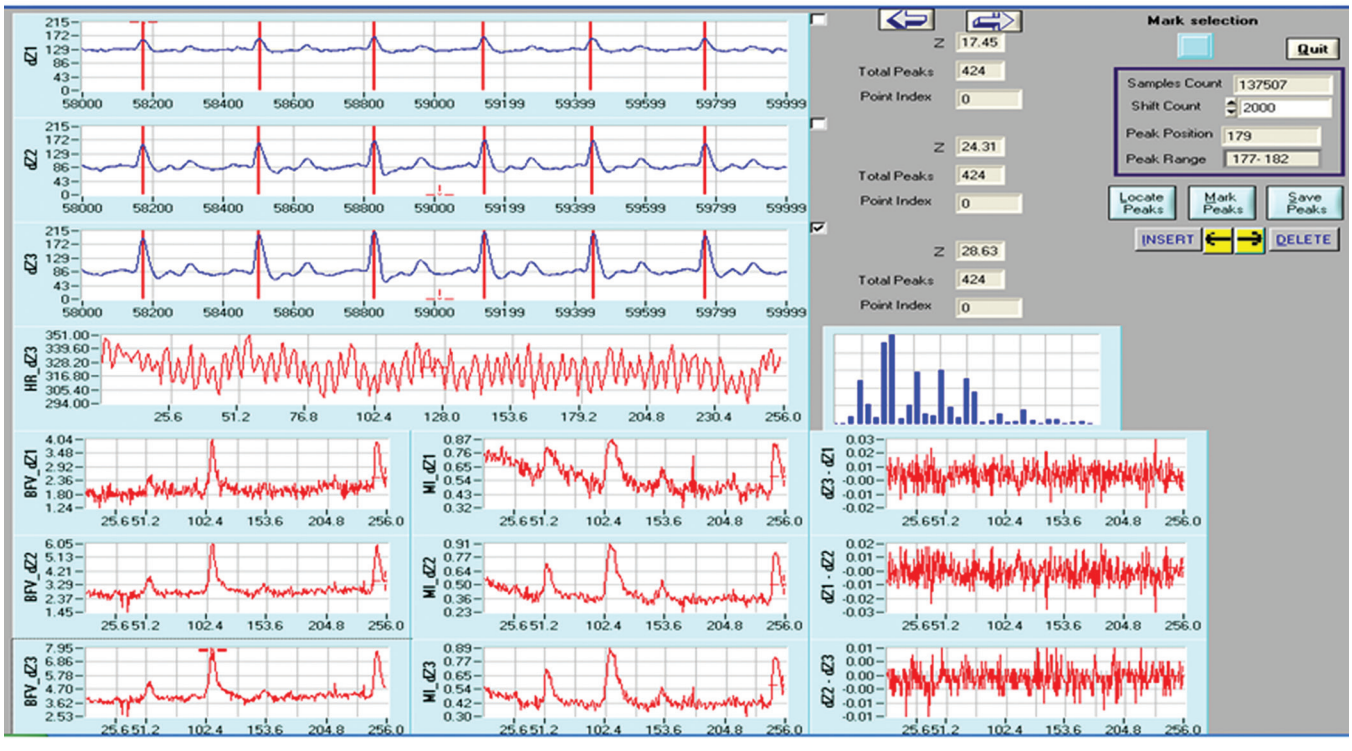


Fig. 5: The selection panel during processing of the data, displaying variability of various parameters in time domain and short-term Fourier transform of a particular data segment

values have been recorded in patients with coronary artery disease (eight in number). These observations suggest that the changes present in coronary arteries are also present in peripheral arteries thereby reducing the arterial compliance and hence the morphology index. Thus, Fourier analysis of peripheral pulse can be used to detect coronary artery disease in early stages.

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REFERENCES

1. Jindal GD, Nerurkar SN, Pedhnekar SA, Babu JP, Kelkar MD, Deshpande AK, Parulkar GB. Diagnosis of peripheral arterial occlusive diseases using impedance plethysmography. J Postgrad Med 1990 Jul;36(3):147-153.
2. Deshpande AK, Jindal GD, Jagasia PM, Murali KV, Bhardwaj PA, Tahlilkar KI, Parulkar GB. Impedance plethysmography of thoracic region: Impedance cardiography. J Postgrad Med 1990 Oct;36(4):207-212.
3. Jindal GD, Babu JP. Calibration of dZ/dt in impedance plethysmography. Med Biol Eng Comput 1985 May;23(3): 279-280.
4. Jindal GD, Nerurkar SN, Pednekar SA, Babu JP, Bhuta AC, Deshpande AK. Corrected formula for estimating peripheral blood flow by impedance plethysmography. Med Biol Eng Comput 1994 Nov;32(6):625-631.
5. Jagruti C, Chaugule N, Ananthakrishnan TS, Babu JP, Jindal GD. Normalized DZ/DT waveform for easy assessment of peripheral blood flow. Proceedings of the symposium on biomedical engineering and nuclear medicine (SBME-NM 2000), BARC, Mumbai, 27-29 January 2000. p. 108-113.
6. Jindal GD, Ananthakrishnan TS, Kataria SK, Deshpande AK. An introduction to impedance cardiovasography. External Report, BARC/2001/E/003, 2001.
7. Jindal GD, Ananthakrishnan TS, Mandlik SA, Sinha V, Jain RK, Kini AR, Naik MA, Kataria SK, Mahajan UA, Deshpande AK. Medical analyzer for the study of physiological variability and disease characterization. External Report BARC/2003/E/012, 2003.
8. Kelkar P, Karamchandani S, Jindal SK. Identifying tridosha in pulse morphology for disease characterization: in a handbook on physiological variability. In: Jindal GD, Deepak KK, Jain RK, editors. Electronics Division BARC; 2010;120-127.
9. Varier PM, Muraleedharan K, Muraleedharan TS, Udayakumari TP, Sudheer AV. Evaluation of peripheral pulse analyzer from Ayurvedic point of view: in A handbook on physiological variability. Jindal GD, Deepak KK, Jain RK, editors. Electronics Division BARC 2010:128-136.
10. Karamchandani SH, Panju M, Mustafa HD, Merchant SN, Desai UB, Jindal GD. Nonlinear and dynamic time warping classification of morphological patterns identified from plethysmographic observations in the radial pulse. Proceedings of 8th International Conference on Information, Communications and Signal Processing (ICICS), IEEE, 13-16 December 2011. p. 1-5.
11. Karamchandani SH, Desai UB, Merchant SN, Jindal GD. Parallel support vector architectures for taxonomy of radial pulse morphology. Signal, Image and Video Processing 2013 Sept;7(5):975-990.
12. Parulkar GB, Jindal GD, Bhardwaj R, Suraokar S, Dharani JB. Impedance cardiography in mitral valve diseases. Indian Heart J 1985 Jan-Feb;37(1):37-42.
13. Jindal GD. Vector impedance cardiography. PhD Thesis. Mumbai: University of Mumbai; 1985. p. 101-105.
14. Malik M. Clinical guide to cardiac autonomic tests. 1st ed. Kluwer Academic Publishers; 1998. p. 423.

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ORIGINAL ARTICLE

Microwave Assisted Biosynthesis of Silver Nanoparticles by Aqueous Extract of Ocimum Sanctum (Tulsi)

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ABSTRACT

Owing to widespread applications, synthesis and characterization of silver nanoparticles is recently attracting considerable attention. Increasing environmental concerns over chemical synthesis routes have resulted in attempts to develop biomimetic approaches. One of them is synthesis using plant parts, which eliminates the elaborate process of maintaining the microbial culture and often found to be kinetically favorable than other bioprocesses.

The present study deals with investigating the effect of process microwave irradiation, interaction time on the morphology and size of silver nanoparticles synthesized using aqueous extract of Tulsi. Plant extract from ocimum sanctum (Tulsi) was used for the synthesis of silver nanoparticles (AgNPs) from silver nitrate solution. Silver nanoparticles were characterized by UV-Vis spectrophotometer and scanning electron microscope (SEM). The formation and stability of the reduced silver nanoparticles in the colloidal solution was monitored by UV-Vis spectrophotometer analysis. Nanoparticles ranges from 20 to 40 nm in size with nearly spherical shape were produced. SEM determination of the brown color stable samples showed the formation of silver nanoparticles and well dispersed nanoparticles could be seen in the samples treated with silver nitrate. These silver nanoparticles have proven to be stable for more than 3 months. It can be inferred from the study that fine tuning the bioprocess parameters will enhance possibilities of desired nano-product tail or made for particular applications.

Keywords: Microwave irradiation, Silver nanoparticles, Size aggregation, Green synthesis, Ocimum sanctum (Tulsi).

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INTRODUCTION

Recently, synthesis of silver nanoparticles has attracted considerable attention owing to their diverse properties like catalysis magnetic and optical polarizability,[1] electrical conductivity,[2] antimicrobial activity[3] and surface enhanced raman scattering (SERS).[4]

A number of synthesis techniques have been developed including chemical reduction of silver ions in aqueous solutions, with or without stabilizing agents,[5] thermal decomposition in organic solvents[6] and chemical and photo reduction in reverse micelles,[7,8] to name a few. Most of these methods are extremely expensive and they also involve the use of toxic, hazardous chemicals which may pose potential environmental and biological risks.

The need for an environmentally sustainable synthesis process has led to the development of some biomimetic approaches. Biomimetics refers to mimicry or imitation of a process. One of the fundamental processes in the biomimetic synthesis involves bio reduction. Recently, a number of inorganic nanomaterials have been synthesized by bio-reduction processes employing different microorganisms. Nanocrystals of gold, silver and their alloys have been synthesized within cells of lactic acid bacteria.[9] But the plant-mediated silver nanoproduct is a relatively newer concept. In this race of AgNP preparation utilizing plants/parts of plants could prove advantageous over other biological processes by eliminating the elaborate process of maintaining the microbial culture and is widely acceptable technology. Existing literature also reports successful synthesis of silver nanoparticles through a green route where the reducing and capping agent selected was the latex obtained from Jatropha curcas.[10] Silver nanoparticles were also obtained using aloe vera,[11] acalypha indica,[12] garcinia mangostana[13] leaf extracts. Crataegus douglasii fruit extract[14] as well as aquous extract of leaves of ocimum sanctum.[15,16] Here we have developed a rapid, eco-friendly and convenient green method for the synthesis of AgNPs from silver nitrate using leaf extracts of Indian medicinal plant, Ocimum sanctum (Tulsi), by microwave irradiation method.

MATERIALS AND METHODS

Preparation of Leaf Extracts Ocimum Sanctum (Tulsi)

The fresh Tulsi leaves were collected from MGMIHS university area and washed with water to remove mud and dust particles. Twenty-five grams of Tulsi leaves was added to100 ml of deionized water and homogenized using a mortar and pestle. The mixture was then boiled at 95°C for 1 hour in a water bath. The mixture was filtered through a Whatman No. 1 filter paper (25 μm). The resulting leaf extract was then stored in refrigerator for further use.

Synthesis of AgNP and Evaluation of Reducing Potential of the Extract

In a typical microwave synthesis, 15 ml Tulsi leaf extract was allowed to interact with 85 ml of 0.001 M $AgNO_3$. It was then placed in a domestic microwave oven (BPL-SANYO BMO-700 TS operating at a power of 1.2 kW and frequency 2450 MHz). The solution was then subjected to microwave heating for 80 seconds. The change of color from light yellow solution to brown solution indicated the formation of silver nanoparticles.

Analysis of Bioreduced Silver Nanoparticles

UV-Vis Spectroscopy

UV-visible spectra were recorded on Shimadzu's UV-Visible Spectrophotometer from 200 to 600 nm. Cuvette of path length 10 mm was used. The measurements were carried out as a function of reaction time at room temperature. The distilled deionized water, adjusted extracts, zero reaction mixture and silver nitrate of 0.01M volume adjusted was used as control to minimize any technical errors.

Scanning Electron Microscopy

Each of the colloidal solution containing AgNPs were centrifuged at 5,000 rpm for 20 minutes and the resulting suspension was redispersed in 10 ml sterile distilled water. The centrifuging and redispersing process was repeated three times. The supernatants were discarded and the final pellets were dissolved in 0.1 ml of deionized water. The pellet was mixed properly and carefully placed on a glass cover slip followed by air-drying. The cover slip itself was used to obtained images of synthesized AgNPs for size and shape determination using the field emission gun-scanning electron microscope (JSM-7600F resolution: 1.0 nm (15 kV), 1.5 nm (1kV); magnification: ×25 to 1,000,000).

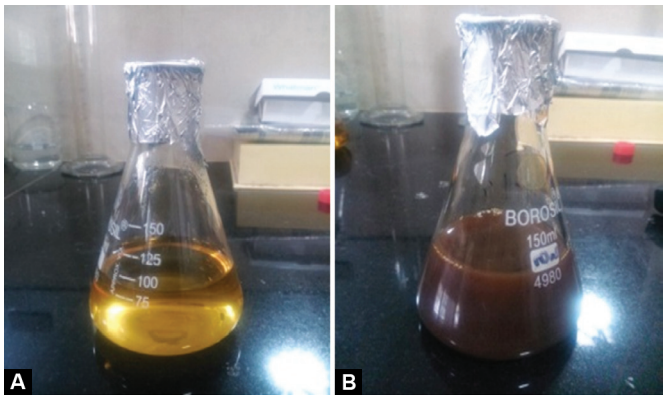
RESULT AND DISCUSSION

Formation of AgNPs by reduction of silver nitrate during exposure to Tulsi leaf extract can be easily monitored from the change in color of the reaction mixture. On adding the aqueous extract of tulsi to $AgNO_3$ solution, the color of their action medium changed rapidly from yellow to brown. The appearance of these colors was due to the excitation of surface plasmon vibrations; typical of silver nanoparticles.[17] The change in color of the reaction mixture within 2 to 3 minutes was obtained, which indicated the formation of AgNPs (Figs 1A and B). This formation indicates that silver ions in reaction medium have been converted to elemental silver having the size of nanometric range. In this study, the formation of silver nanoparticles was initially confirmed using surface plasmon resonance (SPR) phenomenon. For silver nanoparticles, Kmax values were reported in the visible range of 400 to 500 nm.[18]

Figure 2 shows UV-Vis absorption spectrum of silver nanoparticles. Though the plasmon band is broad due to the presence of components in Tulsi extract which are also being read in the spectrophotometric range, it is observed that the silver SPR occurs at 441 nm. There is no change in peak position, suggesting that nucleation of silver nanoparticles starts with initiation of reaction time only, and the size remains unchanged throughout the course of reaction.[19] In the present study, SPR band reveals spherical shape of silver nanoparticles, which was further confirmed by SEM.

Field emission gun-scanning electron microscope results were commensurate with the UV-Visible spectrophotometric analysis. SEM studies were carried out to visualize the size and shape of the AgNPs. Figure 3 shows typical bright field SEM micrograph of the synthesized AgNPs. It was observed that AgNPs were circular or spherical in shape with maximum particles in size range within 20 to 40 nm. An agglomerated silver nanoparticles were observed in many places, thereby indicating possible sedimentation at a later time (Figs 3A and B).

When the metal nanoparticles form in solution, they must be stabilized against the Van der Waals force that may cause coagulation. The stabilization may occur in a number of ways: physisorbed, surfactant and polymers may create steric or electrostatic barriers or purely electrostatic barriers around the particle surface.[20,21] In many cases, the distinction between chemical adsorption (involving direct covalent bonding with the surface metal atoms) and more subtle electrostatic mechanisms (e.g. charge-induced dipole mechanisms and dispersion force mechanisms) is largely a matter of degree. From the present study, it can be stated that microwave irradiation can be an efficient and simple way of producing AgNPs through biological method. More than 90% of the reaction is complete within 2 to 3 minutes of their action time. Generally, biosynthetic methods are considered as time consuming when compared with chemical methods. To the best of our knowledge, reaction time of at least



Figs 1A and B: Color change in reaction mixture (silver nitrate and tulsi extract): (A) Leaf extract and (B) synthesized silver nanoparticles indicated by brown colored solution

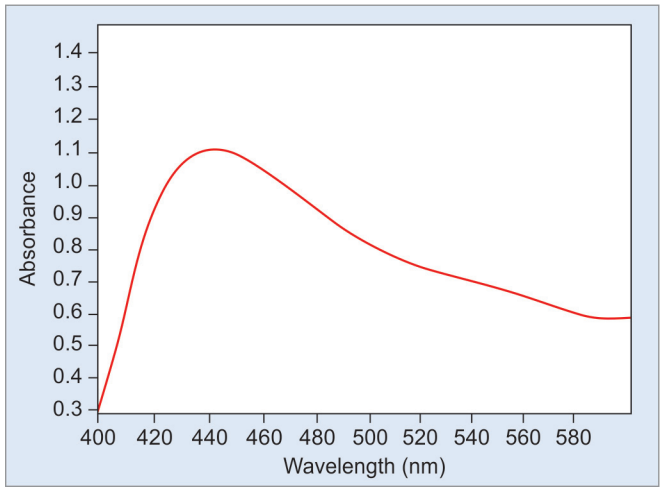
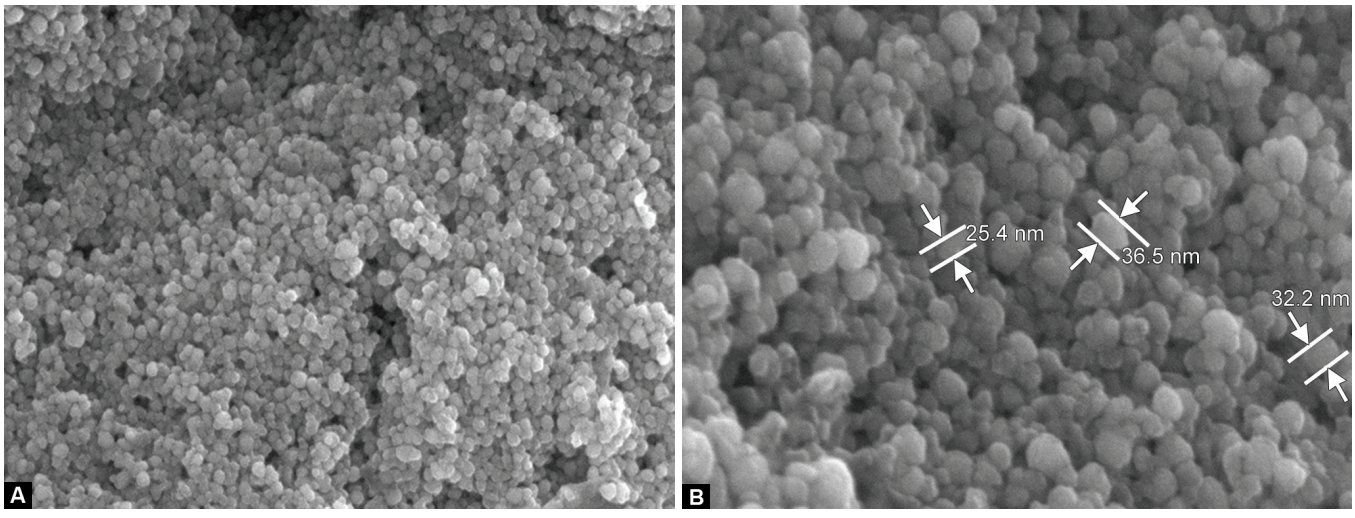


Fig. 2: The UV-visible spectra showing absorption spectra of synthesized silver nanoparticles, with maximum absorption at 441 nm



Figs 3A and B: (A) SEM image shows agglomerated silver nanoparticles at ×60,000 magnification level, (B) SEM image of silver nanoparticles prepared from tulsi extract with diameter of 25, 32 and 36 nm at ×150,000 magnification level

10 to 12 hours is required in plant-mediated nanomaterials synthesis. However, the time consumed in the present study for the reaction to complete is several fold lesser than reported. Such alacrity in reaction time can be the outcome of microwave irradiation of the tulsi extract, which makes the reaction much more efficient than others.

CONCLUSION

The major conclusions drawn from the above study were the following:

1. Formation of AgNPs by reduction of silver nitrate during exposure to tulsi leaf extract can be easily monitored from the change in color of the reaction mixture. Silver nanoparticles bear a characteristic by brown color due to the excitation of surface plasmon vibrations.
2. In a short interaction time of few minutes, highly monodisperse AgNPs silver nanoparticles are synthesized in a range between 20 and 40 nm-size with nearly spherical shape was produced using microwave irradiation. With increasing interaction time (ageing), the aggregation and shape anisotropy of the particles increased.
3. The particles have shown to remain stable for over 3 months.
4. As observed UV-Visible spectrophotometric analysis, bio-organic components from the tulsi extract acted as probable stabilizer for the silver nanoparticles.

In a biological process, fine tuning the process parameters may give products with typical physical characteristics. A detailed study on the mechanistic aspects of the process, i.e. characterizing the reducing and stabilizing contents from the extract is underway.

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REFERENCES

1. Shiraishi Y, Toshima N. Oxidation of ethylene catalyzed by colloidal dispersions of poly (sodium acrylate)-protected silver nanoclusters. Colloids Surf A Physicochemical Eng Asp 2000; 169(1):59-66.
2. Chang LT, Yen CC. Studies on the preparation and properties of conductive polymers VIII: use of heat treatment to prepare metallized films from silver chelate of PVA and PAN. J Appl Polym Sci 1995 Jan;55(2):371-374.
3. Sharverdi AR, Mianaeian S, Shahverdi HR, Jamalifar H, Nohi AA. Rapid synthesis of silver nanoparticles using culture supernatants of enterobacteria: a novel biological approach. Process Biochem 2007;42(5):919-923.
4. Matejka P, Vlckova B, Vohlidal J, Pancoska P, Baumruk V. The role of triton X-100 as an adsorbate and a molecular spacer on the surface of silver colloid: a surface-enhanced Raman scattering study. J Phys Chem 1992;96(3):1361-1366.
5. Liz-Marzan LM, Lado-Tourino I. Reduction and stabilization of silver nanoparticles in ethanol by nonionic surfactants. Langmuir 1996;12(15):3585-3589.
6. Esumi K, Tano T, Torigoe K, Meguro K. Preparation and characterization of bimetallic palladium-copper colloids by thermal decomposition of their acetate compounds in organic solvents. Chem Mater 1990;2(5):564-567.
7. Pileni MP. Fabrication and physical properties of self organized silver nanocrystals. Pure Appl Chem 2000;72(1-2):53-65.
8. Sun YP, Atorngitjawat P, Meziani MJ. Preparation of silver nanoparticles via rapid expansion of water in carbon dioxide micro emulsion into reductant solution. Langmuir 2001;17(19): 5707-5710.
9. Nair B, Pradeep T. Coalescence of nanoclusters and formation of submicron crystallites assisted by lactobacillus strains. Cryst Growth Des 2002;2(4):293-298.
10. Bar H, Bhui DK, Sahoo GP, Sarkar P, De SP, Misra A. Green synthesis of silver nanoparticles using latex of Jatropha curcas. Colloids Surf A Physicochem Eng Asp 2009;339(1-3):134-139.
11. Chandran SP, Chaudhary M, Pasricha R, Ahmad A, Sastry M. Synthesis of gold nanotriangles and silver nanoparticles using Aloe vera plant extract. Biotechnol Prog 2006;22(2):577-583.
12. Krishnaraj C, Jagan EG, Rajasekar S, Selvakumar P, Kalaichelvan PT, Mohan N. Synthesis of silver nanoparticles using acalypha indica leaf extracts and its antibacterial activity against water borne pathogens. Colloids Surf B: Biointerfaces 2010; 76(1):15-20.
13. Veerasamy R, Xin TZ, Gunasagaran S, Xiang TFW, Yang EFC, Jeyakumar N, Dhanaraj SA. Biosynthesis of silver nanoparticles using mangosteen leaf extract and evaluation of their antimicrobial activities. J Saudi Chem Soc 2010;15(2):113-120.
14. Ghaffari-Moghaddam M, Hadi-Dabanlou R. Plant mediated green synthesis and antibacterial activity of silver nanoparticles using crataegus douglasii fruit extract. J Indus Eng Chem 2014; 20(2):739-744.
15. Ramteke C, Chakrabarti T, Sarangi BK, Pandey RA. Synthesis of silver nanoparticles from the aqueous extract of leaves of ocimum sanctum for enhanced antibacterial activity. J Chem 2013;2013:7. Article ID: 278-925.
16. Singhal G, Bhavesh R, Kasariya K, Sharma AR, Sing RP. Biosynthesis of silver nanoparticles using ocimum sanctum (Tulsi) leaf extract and screening its antimicrobial activity. J Nanoparticle Res 2011;13(7):2981-2988.
17. Gardea-Torresdey JL, Gomez E, Peralta-Videa JR, Parsons JG, Troiani M, Jose-Yacaman H. Alfalfa sprouts: a natural source for the synthesis of silver nanoparticles. Langmuir 2003;19(4): 1357-1361.
18. Ankamwar B, Chaudhary M, Sastry M. Gold nanotriangles biologically synthesized using tamarind leaf extract and potential application in vapor sensing. Synth React Inorg Met-Org Nan 2005;35(1):19-26.
19. Ahmad A, Mukherjee P, Senapati S, Mandal D, Khan MI, Kumar R, Sastry M. Extracellular biosynthesis of silver nanoparticles using the fungus fusariums oxysporum. Colloids Surf B 2003; 28(4):313-318.
20. Sastry M, Mayyaa KS, Bandyopadhyay K. pH dependent changes in the optical properties of carboxylic acid derivative silver colloid particles. Colloids Surf A 1997;127(1-3):221-228.
21. Mulvaney P. Surface plasmon spectroscopy of nanosized metal particles. Langmuir 1996;12(3):788-800.

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REVIEW ARTICLE

Potential Role of Estrogen Metabolite 2-Methoxyestradiol in Health and Disease

Amy E Siebert

ABSTRACT

2-Methoxyestradiol (2-ME2) is an endogenous metabolite of 17β-estradiol (E2) that was originally thought to be an inert end product of estrogen metabolism. However, studies conducted over the past two decades have shown 2-ME2 to be a promising anticancer agent. Reports suggest that 2-ME2 directly influences tumor growth through mechanisms which reduce cell proliferation or induce apoptosis as well as through the inhibition of angiogenesis. Incidentally, 2-ME2 as an anticancer agent has poor bioavailability, and this has led to the development of several analogs and derivatives, which currently have had limited success. Thus, it is imperative that we re-evaluate our understanding of 2-ME2-mediated effects in order to innovatively derive, or generate, more efficacious cancer treatment options. In this review, the roles of 2-ME2 in cancer as well as the highly variable mechanisms of action reported for this metabolite are discussed.

Keywords: 2-Methoxyestradiol, Cancer, Mechanism of action, Microtubule disruption, Angiogenesis, Tumor suppressor protein, Estrogen receptor.

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INTRODUCTION

17β-estradiol (E2) is a mitogenic molecule that enhances proliferation in target cells. While several endogenous E2 metabolites have been shown to be more potent estrogenic compounds than their precursor, others, such as 2-methoxyestradiol (2-ME2) (Fig. 1) are nonestrogenic, yet still retain biological activity.[1,2] The formation of 2-ME2 occurs from the catechol O-methyltransferase (COMT)-mediated O-methylation of the catechol estrogen 2-hydroxyestradiol (2-OHE2), a major metabolite formed in humans by the hydroxylation of E2.[3] 2-methoxyestradiol has been shown to have promising therapeutic potential as it displays antiproliferative, antiangiogenic and proapoptotic properties in various cancers while having limited effects on normal cells and tissues.

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2-ME2 and Cancer

Unlike the growth enhancing estrogen metabolites, 2-ME2 has been shown to have potent anti-angiogenic, antiproliferative and pro-apoptotic properties *in vitro* as well as *in vivo*.[4] In contrast to its effects on malignant and transformed cells, 2-ME2 has minimal to no significant effects on the growth of normal cells including lymphocytes.[5-9] There are also indications that 2-ME2 affects actively proliferating cells (with no effect on quiescent cells) possibly due to disruption of cellular events associated with proliferation.[10] For example, Van Zilj et al reported that 2-ME2 disrupted mitotic spindle formation and enhanced Cdc2 kinase activity leading to persistence of the spindle checkpoint. Thus, prolonged metaphase arrest may have resulted in the induction of apoptosis in MCF-7 cells, but not in normal MCF-12A cells.[11]

The most commonly reported effects of 2-ME2 are microtubule disruption, cell cycle arrest, inhibition of angiogenesis, and induction of apoptosis. Several mechanisms have been proposed for 2-ME2 action, but there is a lack of evidence for a common pathway for all of the cells sensitive to this metabolite. The variation in the anti-tumor effects of 2-ME2 reported in the literature is extensive and appears to heavily rely on factors, such as cell type, 2-ME2 concentration, culture conditions, genotype and gene expression profiles. Numerous studies have been conducted in an effort to better understand the biochemical, cellular and molecular mechanisms for the actions of 2-ME2. A portion of these reported effects and mechanisms of action are summarized in the sections below.

MECHANISM OF 2-ME2 ACTION

Estrogen Receptor

Despite being a natural metabolite of E2, the antiproliferative and cytotoxic effects induced by 2-ME2 are independent of estrogen receptor (ER) status and are not considered to be ER-mediated.[12,13] The relative binding affinity of 2-ME2 for ERα and β varies depending on assay conditions.

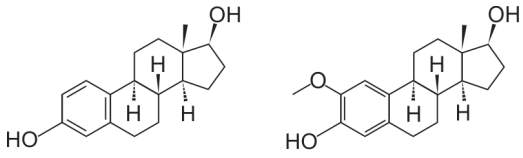

Fig. 1: Structures of 17β-estradiol and 2-methoxyestradiol

Receptor binding assays are a common investigative tool to ascertain the binding specificity and high affinity that would be expected of a hormone receptor. However, factors, such as time, temperature, salt and pH can induce variation and have a detrimental impact on receptor assays *in vitro*.[14] The reported binding affinity of 2-ME2 (relative to estradiol binding) ranges from 0.3 to 2% for ERα and 0.008 to 1% for ERβ.[12,13,15] It is important to note that the biological activity of a steroid hormone may not be accurately determined by receptor binding alone.[14] Liu and Zhu observed both mitogenic and anti-proliferative properties of 2-ME2 in breast cancer cells.[15] The former was reported by the authors to be ER-dependent and occurred at low, nanomolar concentrations (10-750 nM) in the absence of E2 and other growth factors. The observed anti-proliferative effects of 2-ME2 in this study agree with previous findings by other investigators and are reported to be ER-independent. In contrast to the observed results by Liu and Zhu, a previous report from our laboratory has shown that micromolar concentrations of 2-ME2 (1-10 μM) reduces cell number with no observable effects occurring with 1-100 nM 2-ME2 in the ER+ T47D breast cancer cell line.[16]

Microtubule Disruption

Unlike its growth enhancing precursor, 2-ME2 has been reported to interact with microtubules (MTs) to induce mitotic arrest, inhibit cell proliferation and induce apoptosis in tumor cells *in vitro* and *in vivo*.[4,17,18] 2-ME2 binds, in a competitive manner (K_i = 22 μM), at or near the colchicine-binding site of β-tubulin. At high 2-ME2 concentrations, this results in inhibition of tubulin polymerization and MT assembly and subsequent cell cycle arrest at the G2-M transition.[19] In contrast, at low concentrations, 2-ME2-induced mitotic block involves kinetic stabilization of MT dynamics rather than alteration of MT polymerization.[20,21] In fact, it has been shown that low concentrations of 2-ME2 could induce mitotic cell arrest via suppression of MT dynamics and not the deploymerization of MTs.[22]

Inhibition of Angiogenesis

Studies have shown that 2-ME2 is a potent inhibitor of proliferation of transformed and endothelial cells, as well as angiogenesis *in vivo*.[23,24] 2-ME2 is an inhibitor of endothelial cell migration *in vitro* and inhibits the neovascularization of solid tumors, suppressing their growth in mice.[23] The anti-angiogenic effect of 2-ME2 is mediated primarily through inhibition of protein expression, nuclear accumulation and transcriptional activity of hypoxia-inducible factor-1α (HIF-1α). HIF-1α is a transcription factor that stimulates hypoxia-induced secretion of vascular endothelial growth factor (VEGF).[25-27] It was recently reported that 2-ME2 inhibited HIF-1α protein translation by inducing argonaute 2 (Ago2)-mediated association between HIF-1α mRNA and HIF-targeting miRNAs in the cytoplasm. This effect was reported to occur after microtubule disruption and led to the targeted translocation of these complexes to cytoplasmic P-bodies in a microtubule dynamicity-dependent and reversible manner.[28] However, in another report, 2-ME2 was shown to inhibit complex I of the mitochondrial electron transport chain leading to the generation of reactive oxygen species (ROS), which inhibited HIF-1α protein stabilization and mitochondrial respiration in both intact cells and sub-mitochondrial particles.[29]

Cell Cycle Distribution Alterations and Arrest

2-ME2 has been observed to arrest the growth of many human cancer cell lines representing several cell types including Jurkat cells, multiple myeloma, epithelial, melanoma, medulloblastoma cancer cells and transformed fibroblasts at the G2-M transition.[5,9,12,20,30,31] At the biochemical and molecular levels, 2-ME2-induced G2-M cell cycle arrest has been characterized by the induction of cyclin B and Cdc2 kinase activity.[5,20,32] 2-ME2 has also been reported to induce G2-M arrest in breast cancer cell lines regardless of hormone receptor status.[6,32] In other reports, however, the anti-tumor effects of 2-ME2 were not associated with G2-M cell cycle arrest. For example, reports have indicated that 2-ME2 inhibited the growth of pancreatic cancer cells by prolonging S-phase or by inducing both G1-S and G2-M arrest in human osteosarcoma cells and pancreatic cell lines.[33-35] In the ER-positive MCF-7 breast cancer cell line, 2-ME2 (10 nM) induced an increase in cAMP concentration in early S-phase that decreased during mitosis, and phosphorylation of S-phase proteins was enhanced in 2-ME2-exposed cells with no effect on protein synthesis during G2-M transition.[24]

Induction of Apoptotic Cell Death

The induction of apoptosis by 2-ME2 in tumor cells is reported to involve different molecular mechanisms. La Vallee et al have shown that 2-ME2 may utilize the extrinsic pathway for induction of apoptosis.[36] In these studies, the authors reported that 2-ME2 treatment of breast, cervical and prostate carcinoma cells as well as glioma cells and HUVECs

resulted in up-regulation of death receptor 5 (DR5) protein expressions *in vitro* and *in vivo*. This rendered the cells more sensitive to the cytotoxic activities of the DR5 ligand, the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[36] In this study, it was found that 2-ME2-induced apoptosis required sequential activation of caspase-8, caspase-9, and caspase-3. The phosphatidylinositol-3-kinase (PI3K) pathways have also been implicated in 2-ME2-induced activation of the extrinsic apoptotic pathway in prostate cancer cells, and the role of the Akt pathway in the response to 2-ME2 was also explored in human leukemia cells as well.[37,38]

Several studies also suggest that 2-ME2 can induce apoptosis both by tumor suppressor protein p53-dependent and p53-independent mechanisms in various tumor cell types. We have previously shown that in T47D breast cancer cells containing mutant p53, 2-ME2 (1-10 μM) significantly decreases breast cancer cell number and this effect may not be mediated by signaling pathways that are directly influencing, or dependent upon the levels of p53.[16] Interestingly however, concentrations of 2-ME2 that had no significant effect on T47D cell number (1-100 nM) induced p53 protein accumulation which is believed to be due to the possible sequestration of p53 within nucleolar compartments within the cell. In a report by Mukhopadhyah et al, treatment with 5 μM 2-ME2 caused significant growth inhibition of human lung cancer cell lines containing wild-type (wt) p53 (H460 and A549), while having little to no effect on the p53 negative H358 and p53 mutated H322 cell lines.[8] In these studies, the authors found that 2-ME2 treatment up-regulated the endogenous wt p53 protein and subsequently, the cells bypassed the G1-S checkpoint and underwent apoptosis (no change in mutant p53 levels was observed). In four pancreatic cancer cell lines harboring mutant p53, 2 μM 2-ME2 induced S phase arrest and apoptosis that was suggested to occur through a p53-independent mechanism.[35] In another report, 2-ME2 induced G2-M arrest, up-regulated p53 protein levels, and induced micronuclei formation and apoptosis in SV40 T antigen transformed HSF43 lymphoblast cells (line E8T4).[9] In these studies, apoptosis and G2-M block were also observed in two lymphoblast cell lines expressing either low levels of wt p53, or high levels of temperature-sensitive mutant p53, but this was to a much lesser extent than in E8T4 cells and without observed alteration in p53 protein levels.[9] However, when the authors cultured the cells at the permissive temperature, an increase in apoptosis and a prominent G2-M-phase block were present in the mutant p53 cells, suggesting that the high levels of mutant p53 became functional, enhancing the apoptotic effects initiated by 2-ME2.[9]

Further studies have implicated c-jun NH2-terminal kinase (JNK) signaling cascades, including phosphorylation of the anti-apoptotic Bcl-2 family members in 2-ME2-induced apoptosis.[10] In the ER-negative MDA-MB-435s human breast cancer cell line, it was reported that 2-ME2 induced the activation of JNK which was associated with the induction of apoptosis through the mitochondrial pathways as a result of increased phosphorylation (inactivation) of the anti-apoptotic Bcl-2 and Bcl-xL proteins.[39] In comparison, this same study also reported 2-ME2-induced activation of ERK and p38 in these cells, which was found to have a protective effect against 2-ME2-induced apoptosis. In several cell lines derived from prostate, breast, liver and colorectal carcinomas, 2-ME2 treatment led to an activation of JNK and phosphorylation of Bcl-2, which preceded the induction of apoptosis. Thus, it appears that 2-ME2 induces apoptosis in epithelial carcinomas by causing phosphorylation of JNK, which appeared to be correlated with phosphorylation of Bcl-2.[40] However, the stimulus type, regulatory pathways involved and the degree and duration of phosphorylation at specific Bcl-2 residues produce different outcomes.[41] For example, 2-ME2 inhibited the proliferation of Jurkat leukemia cells by up-regulating $p16^{(INK4A)}$ and markedly suppressing the levels of cyclins D3 and E, $p21^{(Cip1/Waf1)}$and E2F1.[41] Further, 2-ME2-induced apoptosis of Jurkat cells was associated with both expression down-regulation as well as JNK-mediated inactivation of Bcl-2, up-regulation of Bak protein levels, activation of caspases-9 and -3 and also PARP-1 cleavage.[41] However, the overexpression of Bcl-2 prevented the 2-ME2-induced apoptotic response by orchestrating a $p27^{(Kip1)}$-dependent G1-S phase arrest which was associated with NF-κB activation.[41] p38/JNK-dependent activation of NF-κB has also been reported to be required for 2-ME2-induced apoptosis in prostate cancer cells, however, a reduction in NF-κB transcriptional and DNA binding activity was observed in 2-ME2-induced apoptosis of medulloblastoma cells.[31,42]

CONCLUSION

The reports summarized within this review clearly demonstrate not only the cell-specific nature of 2-ME2 action, but also genotypic influence on the cellular response to 2-ME2. While this metabolite shows great potential for therapeutic intervention, it is limited by drug disposition challenges.[18] 2-ME2 has poor bioavailability as the rate of oxidation of 2-ME2 is higher than its absorption, limiting the action of the metabolite *in vivo*.[43] Several analogs and derivatives have recently been developed, and while some show promise, they currently lack the ability to induce effects without unwanted cytotoxicity.[43] The specificity for actively

proliferating cancer cells with minimal to no toxicity is an intrinsic property of 2-ME2 that increased its appeal as a treatment modality in the first place. Due to this, it has become imperative that we re-evaluate our understanding of the molecular mechanisms governing the cellular responses to 2-ME2. The need is paramount to more accurately define the necessary factors that are required to be targeted by a drug candidate, in order to successfully achieve desired effects that are similar to those observed with this metabolite. Thus, continued elucidation of the mechanisms of 2-ME2 action is warranted.

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REFERENCES

1. Seegers JC, Aveling ML, Van Aswegen CH, Cross M, Koch F, Joubert WS. The cytotoxic effects of estradiol-17 beta, catecholestradiols and methoxyestradiols on dividing MCF-7 and HeLa cells. J Steroid Biochem 1989;32(6):797-809.
2. Barnea ER, MacLusky NJ, Naftolin F. Kinetics of catechol estrogen-estrogen receptor dissociation: a possible factor underlying differences in catechol estrogen biological activity. Steroids 1983;41(5):643-656.
3. Mannisto PT, Kaakkola S. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacol Rev 1999;51(4):593-628.
4. Pribluda VS, Gubish ER Jr, Lavallee TM, Treston A, Swartz GM, Green SJ. 2-Methoxyestradiol: an endogenous antiangiogenic and antiproliferative drug candidate. Cancer Metastasis Rev 2000;19(1-2):173-179.
5. Kumar AP, Garcia GE, Slaga TJ. 2-methoxyestradiol blocks cell-cycle progression at G2/M phase and inhibits growth of human prostate cancer cells. Molecular Carcinogenesis 2001; 31(3):111-124.
6. Bu S, Blaukat A, Fu X, Heldin N-E, Landström M. Mechanisms for 2-methoxyestradiol-induced apoptosis of prostate cancer cells. FEBS Lett 2002;531(2):141-151.
7. Chauhan D, Li G, Sattler M, Podar K, Mitsiades C, Mitsiades N, Munshi N, Hideshima T, Anderson KC. Superoxide-dependent and -independent mitochondrial signaling during apoptosis in multiple myeloma cells. Oncogene 2003;22(40):6296-6300.
8. Mukhopadhyay T, Roth JA. Induction of apoptosis in human lung cancer cells after wild-type p53 activation by methoxyestradiol Oncogene 1997;14(3):379-384.
9. Seegers JC, Lottering ML, Grobler CJ, van Papendorp DH, Habbersett RC, Shou Y, Lehnert BE. The mammalian metabolite, 2-methoxyestradiol, affects P53 levels and apoptosis induction in transformed cells but not in normal cells. J Steroid Biochemistry Molecular Biol 1997;62(4):253-267.
10. Mueck AO, Seeger H. 2-Methoxyestradiol—Biology and mechanism of action. Steroids 2010;75(10):625-631.
11. Van Zijl C, Lottering ML, Steffens F, Joubert A. In vitro effects of 2-methoxyestradiol on MCF-12A and MCF-7 cell growth, morphology and mitotic spindle formation. Cell Biochem Funct 2008;26(5):632-642.
12. LaVallee TM, Zhan XH, Herbstritt CJ, Kough EC, Green SJ, Pribluda VS. 2-Methoxyestradiol inhibits proliferation and induces apoptosis independently of estrogen receptors alpha and beta. Cancer Res 2002;62(13):3691-3697.
13. Cushman M, He HM, Katzenellenbogen JA, Lin CM, Hamel E. Synthesis, antitubulin and antimitotic activity, and cytotoxicity of analogs of 2-methoxyestradiol, an endogenous mammalian metabolite of estradiol that inhibits tubulin polymerization by binding to the colchicine binding site. J Med Chem 1995;38(12): 2041-2049.
14. Moudgil VK. Progesterone Receptor. In: Agarwal MK, editor. Principles of Recepterology. New York: Walter de Gruyter and Co; 1983. p. 273-379.
15. Liu ZJ, Zhu BT. Concentration-dependent mitogenic and antiproliferative actions of 2-methoxyestradiol in estrogen receptor-positive human breast cancer cells. J Steroid Biochemistry and Molecular Biology 2004;88(3):265-275.
16. Siebert AE, Sanchez AL, Dinda S, Moudgil VK. Effects of estrogen metabolite 2-methoxyestradiol on tumor suppressor protein p53 and proliferation of breast cancer cells. Syst Biol Reprod Med 2011;57(6):279-287.
17. Schumacher G, Neuhaus P. The physiological estrogen metabolite 2-methoxyestradiol reduces tumor growth and induces apoptosis in human solid tumors. J Cancer Res Clin Oncol 2001;127(7):405-410.
18. Verenich S, Gerk PM. Therapeutic promises of 2-methoxyestradiol and its drug disposition challenges. Mol Pharm 2010;7(6): 2030-2039.
19. D'Amato RJ, Lin CM, Flynn E, Folkman J, Hamel E. 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. Proc Natl Acad Sci USA 1994;91(9):3964-3968.
20. Attalla H, Makela TP, Adlercreutz H, Andersson LC. 2-Methoxyestradiol arrests cells in mitosis without depolymerizing tubulin. Biochem Biophys Res Commun 1996;228:467-473.
21. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer 2004;4(4):253-265.
22. Kamath K, Okouneva T, Larson G, Panda D, Wilson L, Jordan MA. 2-Methoxyestradiol suppresses microtubule dynamics and arrests mitosis without depolymerizing microtubules. Molecular Cancer Therapeutics 2006;5(9):2225-2233.
23. Fotsis T, Zhang Y, Pepper MS, Adlercreutz H, Montesano R, Nawroth PP, Schweigerer L. The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. Nature 1994;368(6468):237-239.
24. Lottering ML, Haag M, Seegers JC. Effects of 17 beta-estradiol metabolites on cell cycle events in MCF-7 cells. Cancer Res 1992;52(21):5926-5932.
25. Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW, Giannakakou P. 2-ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 2003;3(4): 363-375.
26. Mooberry SL. Mechanism of action of 2-methoxyestradiol: new developments. Drug Resist Updat 2003;6(6):355-361.

27. Mooberry SL. New insights into 2-methoxyestradiol, a promising antiangiogenic and antitumor agent. Curr Opin Oncol 2003; 15(6):425-430.
28. Carbonaro M, O'Brate A, Giannakakou P. Microtubule disruption targets HIF-1α mRNA to cytoplasmic P-bodies for translational repression. J Cell Biol 2011;192(1):83-99.
29. Hagen T, D'Amico G, Quintero M, Palacios-Callender M, Hollis V, Lam F, Moncada S. Inhibition of mitochondrial respiration by the anticancer agent 2-methoxyestradiol. Biochem Biophys Res Commun 2004;322(3):923-929.
30. Dingli D, Timm M, Russell SJ, Witzig TE, Rajkumar SV. Promising preclinical activity of 2-methoxyestradiol in multiple myeloma. Clin Cancer Res 2002;8(12):3948-3954.
31. Kumar AP, Garcia GE, Orsborn J, Levin VA, Slaga TJ. 2-Methoxyestradiol interferes with NFκB transcriptional activity in primitive neuroectodermal brain tumors: implications for management. Carcinogenesis 2003;24(2):209-216.
32. Zoubine MN, Weston AP, Johnson DC, Campbell DR, Banerjee SK. 2-methoxyestradiol-induced growth suppression and lethality in estrogen-responsive MCF-7 cells may be mediated by down regulation of p34cdc2 and cyclin B1 expression. Int J Oncol 1999;15(4):639-646.
33. Golebiewska J, Rozwadowski P, Spodnik JH, Knap N, Wakabayashi T, Wozniak M. Dual effect of 2-methoxyestradiol on cell cycle events in human osteosarcoma 143B cells. Acta Biochim Pol 2002;49(1):59-65.
34. Qanungo S, Basu A, Das M, Haldar S. 2-Methoxyestradiol induces mitochondria dependent apoptotic signaling in pancreatic cancer cells. Oncogene 2002;21(26):4149-4157.
35. Schumacher G, Kataoka M, Roth JA, Mukhopadhyay T. Potent antitumor activity of 2-methoxyestradiol in human pancreatic cancer cell lines. Clin Cancer Res 1999;5(3):493-499.
36. LaVallee TM, Zhan XH, Johnson MS, Herbstritt CJ, Swartz G, Williams MS, Hembrough WA, Green SJ, Pribluda VS. 2-methoxyestradiol up-regulates death receptor 5 and induces apoptosis through activation of the extrinsic pathway. Cancer Res 2003;63(2):468-475.
37. Shimada K, Nakamura M, Ishida E, Kishi M, Matsuyoshi S, Konishi N. The molecular mechanism of sensitization to Fas-mediated apoptosis by 2-methoxyestradiol in PC3 prostate cancer cells. Molecular Carcinogenesis 2004;39(1):1-9.
38. Gao N, Rahmani M, Dent P, Grant S. 2-Methoxyestradiol-induced apoptosis in human leukemia cells proceeds through a reactive oxygen species and Akt-dependent process. Oncogene 2005;24(23):3797-3809.
39. Fukui M, Zhu BT. Mechanism of 2-methoxyestradiol-induced apoptosis and growth arrest in human breast cancer cells. Mol Carcinog 2009;48(1):66-78.
40. Bu S, Blaukat A, Fu X, Heldin N-E, Landström M. Mechanisms for 2-methoxyestradiol-induced apoptosis of prostate cancer cells. FEBS Letters 2002;531(2):141-151.
41. Batsi C, Markopoulou S, Kontargiris E, Charalambous C, Thomas C, Christoforidis S, Kanavaros P, Constantinou AI, Marcu KB, Kolettas E. Bcl-2 blocks 2-methoxyestradiol induced leukemia cell apoptosis by a p27(Kip1)-dependent G1/S cell cycle arrest in conjunction with NF-κB activation. Biochem Pharmacol 2009;78(1):33-44.
42. Shimada K, Nakamura M, Ishida E, Kishi M, Konishi N. Roles of p38- and c-jun NH2-terminal kinase-mediated pathways in 2-methoxyestradiol-induced p53 induction and apoptosis. Carcinogenesis 2003;24(6):1067-1075.
43. Pinto MP, Medina RA, Owen GI. 2-Methoxyestradiol and disorders of female reproductive tissues. Horm Cancer 2014 Apr 25. [Epub ahead of print].

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REVIEW ARTICLE

Hand-made Cloning: A Guide for Cloning Water Buffaloes

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ABSTRACT

The present review is a detailed discussion on comparable benefits of hand-made cloning (HMC) technique than micromanipulation based conventional cloning and developed in the author's laboratory. Hand-made cloning technique does not require micromanipulators, because the manipulations required for both enucleation and nucleus transfer are performed by hand. The HMC technique includes manual bisection of zona-free oocytes and the simultaneous fusion of the somatic cell with two cytoplasts to produce a cloned embryo. The benefits of HMC include low setup costs for limited equipment, no requirement of highly trained expertise and *in vitro* efficiency comparable to traditional somatic cell nuclear transfer technology. Embryos produced by HMC can be cryopreserved and capable of producing live births. The HMC technique is now applied to different species and can be used in large scale nuclear transfer programs.

Keywords: Buffalo, Cloning, Hand-guided cloning, Somatic-cell.

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INTRODUCTION

It is now approximately two decades since the birth, in 1996, of Dolly the first farm animal to be produced by nuclear transfer using an adult derived somatic cell as nuclear donor. The cloning of mammals by nuclear transfer is commonly regarded as a revolutionary approach and the ultimate cutting-edge technology; however, the principles were outlined 70 years ago.[1] At that time for mammalian nuclear transfer this technology was used by the most laboratories, published 1986.[2] With slight improvement to make enucleation more accurate,[3] this somatic cell nuclear transfer (SCNT) technique was subsequently adapted without significant changes for somatic cell nuclear transfer.[4]

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The first live offspring produced from differentiated cell populations were two lambs born in 1995 using cultured embryonic cells as nuclear donors and enucleated unfertilized eggs (metaphase II oocytes (MII)) as recipient cytoplasts.[5] In the following year offspring were produced using cultured cell populations derived from fetal and adult tissues.[6] Since this time SCNT has been successfully applied to a range of species including cattle,[7] mice,[8] goats,[9] pigs,[10] cats,[11] rabbits,[12] horses,[13] rats,[14] dogs,[15] ferrets[16] and buffaloes[17] using a range of cell types.

Somatic cell nuclear transfer offers a range of opportunities in basic and applied research, in agriculture and wildlife conservation.[18] However, to fulfill much of this potential a simple, repeatable and robust methodology is required. Over the last two decades more than 99% of scientific publications dealing with somatic cell cloning are based on micromanipulation-based enucleation and nuclear transfer. Consequently, nuclear transfer remained the privilege of selected laboratories that could afford the considerable investment regarding both instrumentation and skills. As a consequence, over all costs are very high and financing of this type of research frequently requires commercial contribution.

ALTERNATIVE APPROACH

From a technical point of view, there is very little change in nuclear transfer methods during the past 20 years. Only a small (but growing) group of scientists have been looking for different technical solutions; and after many dead ends, the new route is now, more or less, outlined and might offer a real alternative. The main element of this new approach is a simplification of a process, the decrease in the requirements of time and investment and skilled workforce. The results achieved are at least competitive with those of the commonly used nuclear transfer procedure—traditional cloning (TC).[19]

Advantages of Hand-made Cloning

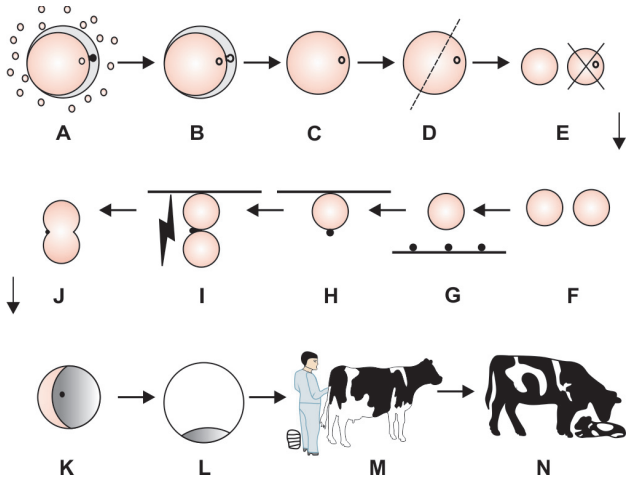
1. *Equipment*: Less expensive than that required for micromanipulation-based cloning.
2. *Procedure*: Simple, rapid, easy to learn and perform.
3. *Efficiency*: Required time, workforce and investment are lower than in traditional cloning. Transferable embryo per oocyte rates are approximately the same, although two oocytes are used for reconstruction of one embryo.

4. *Embryo cryopreservation*: Possible to produce healthy offspring produced in cattle and pig.
5. *Pregnancy and calving and/or furrowing rates*: According to the few available data, at least identical with those reported after micromanipulation-based traditional cloning.
6. *Special benefits*: Possibility for automation with the microchannel-microfluidics technology.

For the normal development of mammalian embryo, there is general assumption that zona pellucida is very important. This assumption has restricted the creative thinking to improve the *in vitro* reproductive technology until some attempts have been made to break this assumption. Evidence regarding the possibility of zona-free *in vitro* fertilization[20,21] and parthenogenesis activation and embryo culture[22,23] in cattle and pigs have incrementally opened the way for zona-free manipulations. The first known zona-free nuclear transfer approach was performed by Tatham et al.[24] Unfortunately, their method for enucleation (density-gradient centrifugation of zona-free oocytes) was unreliable and no calves were obtained after fusion with embryonic cells.

In the 20th century, the only known successful attempt to exclude micromanipulators from the mammalian nuclear transfer procedure was that of Peura et al[25] resulting in healthy offspring.[26] The idea to perform enucleation by oriented or random manual bisection of oocytes was based on earlier embryo bisection[27-29] and blastocyst biopsy techniques.[30] The invention of handmade enucleation with a sharp blade established a reliable system for reconstruction by fusing two enucleated oocytes to one blastomere Peura et al.[25] With slight modifications of the original technique zona-free nuclear transfer methods for somatic cell cloning in cattle and pig was successfully done. This technique of Vajta et al[31] was performed entirely by hand without sophisticated tools this is where the name hand-made cloning (HMC) originated from.[32-34]

Hand-made cloning is a very simple process (Figs 1Ato N). The somatic cell was glued to the surface of the enucleated cytoplast with phytohemagglutinin before fusion, and the reconstructed embryos were placed, individually, into microwells[31,35] for culture. Microblade is used to enucleate the oocyte in HMC technique. One-third of the cytoplasm containing an extrusion cone was removed. The efficiency of enucleation by using HMC is effective and reliable (98%).[36] Time and productivity are crucial factors in cloning, not only to decrease the costs but also to increase the quality of the produced embryos. Most researchers cloners agree that the time oocytes, cytoplasts and embryos spend outside the incubator inversely correlates with their quality. Selokar et al have optimized electrofusion conditions and post holding time of embryos for efficient production of



Figs 1A to N: Schematic illustration of hand-made cloning: (A) cumulus-intact oocytes 18 to 22 hours after maturation, (B) cumulus removal by vortexing, (C) zonae removal, (D) bisection of oocytes, (E) Hoechst 33342 staining, (F) selection of cytoplasts, (G and H) attachment of somatic cell to the cytoplast, (I) simultaneous fusion of two cytoplasts with a somatic cell, (J) round-up following fusion, (K) activation of embryos 3 hours after reconstruction, (L) blastocyst 7-day after reconstruction, (M) embryo transfer with fresh and/or vitrified embryos and (N) offspring

zona-free nuclear transfer embryos in buffalos (*Bubalus bubalis*).[37] Cytoplasm volume may also play crucial role in development of blastocyst. Panda et al demonstrated that affects of cytoplasm volume on the developmental competence of hand-made cloned buffalo embryos.[38]

DONOR CELL TYPE AND AGE

After the production of the first mammals from cultured embryonic[5] fetal and adult cell lines[4] numerous studies provided extensive evidence that somatic cells could be used to produce cloned offspring. Subsequently, numerous somatic cell types including mammary epithelial,[4] ovarian cumulus cells,[39] fibroblasts,[40] sertoli cells,[41] lymphocytes, natural killer T cells,[42] mature B and T cells, olfactory, neural stem cells and myoblasts[43] have be used for SCNT[44,45] indicating that different tissues and cells from donors of different ages can be reprogramed. Some cells types appear to be more reprogramable than others. Tian et al used bovine cumulus cells, fibroblast and mammary epithelial cells to generate NT calves to assess the differences between cell types.[46] However, Ogura et al demonstrated no significant difference between cumulus cells, fibroblasts and sertoli cells in their ability to support full-term development.[41] George et al produced a cloned buffalo calf using buffalo ES cell-like cell as a donor.[47] Golla et al used somatic cells isolated from milk for the production of cloned embryo. Somatic cells in milk are a potential source of nuclei for nuclear transfer to produce genetically identical animals; this is especially important in animals that are susceptible to risks of bacterial infection on biopsy collection.[17]

Pluripotent cells, such as embryonic blastomeres and embryonic stem cells support development of nuclear transfer embryos at a higher efficiency than somatic cells.[48,49] Muzaffar et al demonstrated that ESCs derived from blastocysts produced by parthenogenesis or HMC could be possible alternatives to those derived from blastocysts produced by IVF, because their similarity was established by immunocytochemistry, expression of pluripotency genes, and differentiation potential.[50] The cell cycle stage of both the donor nucleus and recipient cytoplast at the time of transfer are important whether by fusion or injection and can affect both the efficiency of transfer and also subsequent nuclear reprogramming.[51,52] Selokar et al showed that buffalo fibroblast cells can be synchronized at the G0/G1 stage using total confluence, serum starvation or roscovitine treatment.[53]

ACTIVATION OF OOCYTES AND RECONSTRUCTED COUPLETS

The activation process is very important for the development of reconstructed embryos in HMC. Ionomycin or calcium ionophore combined with 6-dimethylaminopurine (6-DMAP) or cycloheximide is one of the most widely used activation protocols for reconstructed oocyte after nuclear transfer.[54-56] George et al demonstrated that zona-free buffalo oocytes can be successfully activated for parthenogenetic development using chemical or electrical stimulation. Out of different agents examined, chemical activation by CaI followed by 6-DMAP resulted in the highest blastocyst rate.[57] Ionomycin exclusively mobilizes intracellular Ca^{2+} stores to induce only a single calcium release rather than a repetitive series as occurs naturally.[58] Calcium inactivates CSF suppressing activity of the maturation promoting factor (MPF), followed by administration of chemicals, such as 6-dimethylaminopurine (6-DMAP) a serine protease inhibitor to suppress or prevent reformation of MPF activity.[59-62] To determine the best activation protocol in ovine reconstructed oocyte, Loi et al compared various chemical treatments and embryonic development.[55]

CULTURE AND TRANSFER OF HMC EMBRYOS

The overall *in vitro* efficiency of HMC is similar to traditional nuclear transfer and are identical to or even better than the results of *in vitro* fertilization experiments performed in parallel[63-67] in cattle and pigs. HMC system is capable of producing approximately 50% blastocyst rates, among the highest described for somatic cell cloning in cattle.[32,33] If we compare the quality of blastocyst, the only difference between zona-free and traditional cloning may be the slightly higher cell number in the embryos derived from the zona-free system[32,67] for cattle and pigs, respectively. The inner cell mass (ICM) of the HMC cattle embryos is usually well defined and represents approximately 30% of the total cell number.[32] Limited ultra-structural analysis of HMC blastocysts did not show any remarkable morphological difference compared with those produced by *in vitro* fertilization or derived after *in vivo* embryo production.[32]

PREGNANCY AND CALVING RATES

The transfer of zona-free embryo to surrogate does not present a technical challenge. In fact, it might help to overcome the problems related to hatching, which are aggravated by the zona hardening as a consequence of *in vitro* embryo culture. Pregnancy rates of approximately 50% can be achieved with cloned zona-free embryos, both in cattle and pigs.[68-70] According to the limited available data, no significant difference in the rate of developmental anomalies between TC and HMC was observed in cattle. Similar observations were published regarding transfer of cloned zona-free embryos in horse and mouse.[69,71,72] Zona-free cloned blastocysts could be successfully cryopreserved by vitrification and used to obtain live offspring in buffalo. Saha et al successfully produced cloned calves from vitrified warmed zona-free buffalo (Bubalus bubalis) embryos by HMC.[73]

EPIGENETIC REPROGRAMMING

In vitro produced mammalian embryos differ from their *in vivo* counterparts. These embryos are sensitive to environmental conditions that can affect embryo morphology, gene expression, embryonic growth and developmental potential both pre- and postnatal.[74,75] Evidence traditionally indicates that mammalian embryos display environmental sensitivity to *in vitro* procedures which manifest in phenotypic condition known as large offspring syndrome (LOS[76,77]). LOS is identified by obvious abnormalities, such as increased incidence of oversize fetuses and calves, increased fetal myogenesis, dystocia, dysfunctional perinatal pulmonary activity, abnormalities in placental development and reduced pregnancy rates.[74,75] The developmental effects of nuclear transfer observed in terms of blastocyst formation, implantation, development to term and postnatal survival are thought to be associated with faulty epigenetic reprogramming of donor nuclei leading to aberrant expression of key developmental genes.[78-80] The occurrence of LOS is due to alteration of epigenetic patterns associated with preimplantation embryo chromatin[78,81,82] resulting in altered expression of imprinted and nonimprinted genes.[83-87] It has been recently demonstrated that global hypomethylation of a differentiated cell genome prior to SCNT

improved cloning efficiency.[88] In addition, differences in the methylation status of histone H3 at lysines 4 (H3K4) and 9 (H3K9) and 27 (H3K27) between quiescent and cycling murine B lymphocytes are linked to development. Methylation is markedly reduced at all three positions in quiescent lymphocytes which have a correspondingly greater developmental potential following SCNT.[89]

FUTURE WAY OF HMC

As discussed, HMC technology is very easy to adopt with less investment is required on equipment and is time-saving methodology. The latter can be applied in many fields like agricultural-livestock, wild life conservation and interspecies cloning. One more step to wild life conservation, Priya et al have successfully produced wild buffalo embryos by interspecies somatic cell nuclear transfer (iSCNT) through HMC using wild buffalo somatic cells and oocytes of domestic buffalo (Bubalus bubalis).[18] One can attempt to restore dead valuable animals through HMC. Our findings pave the way for restoration of highly precious progeny-tested buffalo-bulls, which has immense economic importance, and can also be used for restoration of endangered species Selokar et al.[90]

CONCLUSION

None of the comparisons between HMC with traditional cloning has proved the inferiority of zona free cloning *v.* micromanipulation-based traditional nuclear transfer. Additionally, advantages of HMC in limited requirements for equipment, skilled workforce and time invested both for education/training and production gives a definite place for this method in future nuclear transfer research and practice. The main benefit of this new approach is that it offers a simpler way for somatic cell cloning. This HMC method is so simple that one can easily standardize, transfer the technique from one laboratory to another without significant changes and variations so that differences between laboratories and scientist can be minimized. Eventually this will help in more rapid advancement in the research of somatic cell nuclear transfer.

REFERENCES

1. Spemann H. Embryonic development and induction. London: Yale University Press;1938. p 211.
2. Willadsen SM. Nuclear transplantation in sheep embryos. Nature 1986;320(6057):63-65.
3. Westhusin ME, Levanduski MJ, Scarborough R, Looney CR, Bondioli KR. Viable embryos and normal calves after nuclear transfer into Hoechst stained enucleated demi-oocytes of cows. J Reprod Fertil 1992;95(2):475-480.
4. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997;385(6619):810-813.
5. Campbell KHS, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996; 380(6569):64-66.
6. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. Nature 1997;385(6619):810-813.
7. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, et al. Cloned transgenic calves produced from non-quiescent fetal fibroblasts. Science 1998;280(5367):1256-1258.
8. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998;394(6691): 369-374.
9. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, et al. Production of goats by somatic cell nuclear transfer. Nat Biotechnol 1999;17(5):456-461.
10. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, et al. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 2000;407(6800):86-90.
11. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M. Cell biology: a cat cloned by nuclear transplantation. Nature 2002 Feb 21;415(6874):859.
12. Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat Biotechnol 2002;20(4):366-369.
13. Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, et al. Pregnancy: a cloned horse born to its dam twin. Nature 2003;424(6949):635.
14. Zhou Q, Renard JP, Le Friec G, Brochard V, Beaujean N, Cherifi Y, et al. Generation of fertile cloned rats by regulating oocyte activation. Science 2003;302(5648):1179.
15. Lee BC, Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, et al. Dogs cloned from adult somatic cells. Nature Aug 2005; 436(7051):641.
16. Li Z, Sun X, Chen J, Liu X, Wisely SM, Zhou Q, et al. Cloned ferrets produced by somatic cell nuclear transfer. Dev Biol 2006 May;293(2):439-448.
17. Golla K, Selokar NL, Saini M, Chauhan MS, Manik RS, Palta P, Singla SK. Production of nuclear transfer embryos by using somatic cells isolated from milk in buffalo (Bubalus bubalis). Reprod Domest Anim 2012;47(5):842-848.
18. Priya D, Selokar Nl, Raja AK, Saini M, Sahare A, Nala N, Palta P, Chauhan MS, Manik RS, Singla SK. Production of wild buffalo (bubalus arnee) embryos by interspecies somatic cell nuclear transfer using domestic buffalo (bubalus bubalis) oocytes. Reprod Domest Anim 2014;49(2):343-351.
19. Vajta G. Handmade cloning: the future way of nuclear transfer? Trends Biotechnol 2007;25(6):250-253.
20. Xu KP, et al. Immature zona-free bovine oocytes can be matured, fertilized and cultured to the blastocyst stage in vitro. J Reprod Fertil 1990;5:57.
21. Wu GM et al. Birth of piglets by in vitro fertilization of zona free porcine oocytes. Theriogenology 2004;62(8):1544-1556.
22. Vajta G, et al. New method for culture of zona-included or zona free embryos: the well-of-the-well (WOW) system. Mol Reprod Dev 2000 Mar;55(3):256-264.
23. Booth PJ, et al. Application of the zona-free manipulation technique to porcine somatic nuclear transfer. Cloning Stem Cells 2000;3(4):191-197.
24. Tatham BG, et al. Enucleation by centrifugation of in vitro matured bovine oocytes for use in nuclear transfer. Biol Reprod 1995 Nov;53(5):1088-1094.

25. Peura TT, Lewis IM, Trounson AO. The effect of recipient oocyte volume on nuclear transfer in cattle. Mol Reprod Dev 1998 Jun;50(2):185-191.
26. Peura TT, Lane MW, Lewis IM, Trounson AO. Development of bovine embryo-derived clones after increasing rounds of nuclear recycling. Mol Reprod Dev 2001 Apr;58(4):384-389.
27. Rorie RW, McFarland CW, Overskei TL, Voelkel SA, Godke RA. A new method of splitting embryos without the use of a commercial micromanipulation unit. Theriogenology 1985; 23:224.
28. Maurer RR. Embryo splitting and transfer in sheep. Theriogenology 29:276.
29. Williams J, Moore L. Quick-splitting of bovine embryos. Theriogenology 1988 Feb;29(2):477-484.
30. Bredbacka P, Kankaanpää A, Peippo J. PCR-sexing of bovine embryos: a simplified protocol. Theriogenology 1995 Jul;44(2): 167-176.
31. Vajta G, Lewis IM, Hyttel P, Thouas GA, Trounson AO. Somatic cell cloning without micromanipulators. Cloning 2001;3(2): 89-95.
32. Vajta G, Lewis IM. Trounson AO, Purup S, Maddox-Hyttel P, Schmidt M, Pedersen HG, Greve T, Callesen H. Handmade somatic cell cloning in cattle: analysis of factors contributing to high efficiency in vitro. Biol Reprod 2003 Feb;68(2):571-578.
33. Vajta G, Peura TT, Lai L, Murphy CN, Prather RS, Kragh PM, Holm P, Callesen H. Highly efficient and reliable chemically assisted enucleation method for handmade cloning (HMC) in cattle and pig. Reprod Fertil Dev 2004 Jan;16(2):159.
34. Vajta G, Bartels P, Joubert J, de la Rey M, Treadwell R, Callesen H. Production of a healthy calf by somatic cell nuclear transfer without micromanipulators and carbon dioxide incubators using the handmade cloning (HMC) and the submarine incubation system (SIS). Theriogenology 2004 Nov;62(8): 1465-1472.
35. Thouas GA, et al. The GO system—a novel method for microculture for in vitro development of mouse zygotes to the blastocyst stage. Reproduction 2003 Aug;126(2):161-169.
36. Du Y, Kragh PM, Zhang X, Purup S, Yang H, Bolund L, et al. High overall in vitro efficiency of porcine handmade cloning (HMC) combining partial zona digestion and oocyte trisection with sequential culture. Cloning Stem Cells 2005;7(3):199-205.
37. Selokar NL, Shah RA, Saha AP, Muzaffar M, Saini M, Chauhan MS, Manik RS, Palta P, Singla SK. Effect of post-fusion holding time, orientation and position of somatic cell-cytoplasts during electrofusion on the development of handmade cloned embryos in buffalo (Bubalus bubalis). Theriogenology 2012 Sep;78(4):930-936.
38. Panda SK, George A, Saha AP, Sharma R, Manik RS, Chauhan MS, Palta P, Singla SK. Effect of cytoplasmic volume on developmental competence of buffalo (Bubalus bubalis) embryos produced through hand-made cloning. Cell Reprogram: 2011 Jun;13(3):257-262.
39. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998 Jul;394(6691): 369-374.
40. Kasinathan P, Knott JG, Wang Z, Jerry DJ, Robl JM. Production of calves from G1 fibroblasts. Nat Biotechnol 2001 Dec; 19(12):1176-1178.
41. Ogura A, Inoue K, Ogonuki N, Noguchi A, Takano K, Nagano R, et al. Production of male cloned mice from fresh, cultured, and cryopreserved immature sertoli cells. Biol Reprod 2000 Jun; 62(6):1579-1584.
42. Inoue K, Wakao H, Ogonuki N, Miki H, Seino K, Nambu-Wakao R, et al. Generation of cloned mice by direct nuclear transfer from natural killer T cells. Curr Biol 2005 Jun;15(12):1114-1118.
43. Gao S, McGarry M, Latham KE, Wilmut I. Cloning of mice by nuclear transfer. Cloning Stem Cells 2003;5(4):287-294.
44. Shiga K, Fujita T, Hirose K, Sasae Y, Nagai T. Production of calves by transfer of nuclei from cultured somatic cells obtained from Japanese black bulls. Theriogenology 1999 Aug; 52(3):527-535.
45. Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J Reprod Fertil 2000 Nov;120(2):231-237.
46. Tian XC, Kubota C, Enright B, Yang X. Cloning animals by somatic cell nuclear transfer—biological factors. Reprod Biol Endocrinol 2003;1:98.
47. George A, Sharma R, Singh KP, Panda SK, Singla SK, Palta P, Manik R, Chauhan MS. Production of cloned and transgenic embryos using buffalo (Bubalus bubalis) embryonic stem cell-like cells isolated from in vitro fertilized and cloned blastocysts. Cell Reprogram 2011 Jun;13(3):263-272.
48. Eggan K, Rode A, Jentsch I, Samuel C, Hennek T, Tintrup H, et al. Male and female mice derived from the same embryonic stem cell clone by tetraploid embryo complementation. Nat Biotechnol 2002 May;20(5):455-459.
49. Zhou Q, Jouneau A, Brochard V, Adenot P, Renard JP. Developmental potential of mouse embryos reconstructed from metaphase embryonic stem cell nuclei. Biol Reprod 2001 Aug; 65(2):412-419.
50. Muzaffar M, Selokar NL, Singh KP, Zandi M, Singh MK, Shah RA, Chauhan MS, Singla SK, Palta P, Manik R, et al. Equivalency of buffalo (Bubalus bubalis) embryonic stem cells derived from fertilized, Parthenogenetic, and hand-made cloned embryos. Cell Reprogram 2012 Jun;14(3):267-279.
51. Campbell KHS. Nuclear equivalence, nuclear transfer, and the cell cycle. Cloning 1999;1(1):3-15.
52. Campbell KHS, Alberio R. Reprogramming the genome: role of the cell cycle. Reprod Suppl 2003;61:477-494.
53. Selokar NL, Saini M, Muzaffer M, Krishnakanth G, Saha AP, Chauhan MS, Manik R, Palta P, Madan P, Singla SK, et al. Roscovitine treatment improves synchronization of donor cell cycle in G0/G1 stage and in vitro development of handmade cloned buffalo (Bubalus bubalis) embryos. Cell Reprogram 2012 Apr;14(2):146-154.
54. Akagi S, Adachi N, Matsukawa K, Kubo M, Takahashi S. Developmental potential of bovine nuclear transfer embryos and postnatal survival rate of cloned calves produced by two different timings of fusion and activation. Mol Reprod Dev 2003 Nov;66(3):264-272.
55. Loi P, Ledda S, Fulka J, Cappai P, Moor RM. Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. Biol Reprod 1998 May;58(5):1177-1187.
56. Wells DN, Misica PM, Tervit HR. Production of cloned calves following nuclear transfer with cultured adult mural granulose cells. Biol Reprod 1999 Apr; 60(4):996-1005.
57. George A, Shah RA, Sharma R, Palta P, Singla SK, Manik RS, Chauhan MS. Activation of zona-free buffalo (Bubalus bubalis) oocytes by chemical or electrical stimulation, and subsequent parthenogenetic embryo development. Reprod Domest Anim 2011 Jun;46(3):444-447.
58. Hoth M, Penner R. Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature1992;355:353-356.

59. Susko-Parrish JL, Leibfried-Rutledge ML, Northey DL, Schutzkus V, First NL. Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. Dev Bio 1994 Dec;166(2):729-739.
60. Verlhac MH, De Pennart H, Maro B, Cobb MH, Clarke HJ. MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. Dev Bio 1993 Aug;158(2):330-340.
61. Yang X, Presicce GA, Moraghan L, Jiang S, Foote RH. Synergistic effect of ethanol and cycloheximide on activation of freshly matured bovine oocytes. Theriogenology 1994 Feb;41(2): 395-403.
62. Szollosi MS, Kubiak JZ, Debey P, De Pennart H, Szollosi D, Maro B. Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes. J Cell Sci 1993 Mar;104(pt 3):861-872.
63. Booth PJ, Tan SJ, Reipurth R, Holm P, Callesen H. Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique. Cloning Stem Cells 2001;3(3): 139-150.
64. Oback B, Wells DN. Cloning cattle. Cloning Stem Cells 2003;5(4):243-256.
65. Oback B, Wiersema AT, Gaynor P, Laible G, Tucker FC, et al. Cloned cattle derived from a novel zona-free embryo reconstruction system. Cloning Stem Cell 2003;5(1):3-12.
66. Wells DN, Laible G, Tucker FC, Miller AL, Oliver JE, et al. Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. Theriogenology 2003 Jan;59(1):45-59.
67. Kragh P, Vajta G, Corydon TJ, Purup S, Bolund L, Callesen H. Production of transgenic porcine blastocysts by hand-made cloning. Reprod Fertil Dev 2004;16(3);315-318.
68. Lagutina I, et al. Birth of cloned pigs from zona-free nuclear transfer blastocysts developed in vitro before transfer. Cloning Stem Cells 2006;8(4):283-293.
69. Lagutina I, et al. Comparative aspects of somatic cell nuclear transfer with conventional and zona-free method in cattle, horse, pig and sheep. Theriogenology 2007 Jan;67(1):90-98.
70. Du Y, et al. Piglets born from handmade cloning. Reprod Fertil Dev 2007;19(1):135-136.
71. Lagutina I, et al. Somatic cell nuclear transfer in horses: effect of oocyte morphology, embryo reconstruction method and donor cell type. Reproduction 2005 Oct;130(4):559-567.
72. Ribas R, et al. Modifications to improve the efficiency of zona-free mouse nuclear transfer. Cloning Stem Cells 2006;8(1): 10-15.
73. Saha A, Panda SK, Chauhan MS, Manik RS, Palta P, Singla SK. Birth of cloned calves from vitrified-warmed zona-free buffalo (Bubalus bubalis) embryos produced by hand-made cloning. Reprod Fertil Dev 2013;25(6):860-865.
74. Summers MC, Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. Hum Reprod Update 2003 Nov-Dec;9(6):557-582.
75. Fleming TP, KwongWY, Porter R, Ursell E, Fesenko I,Wilkins A, et al. The embryo and its future. Biol Reprod 2004 Oct;71(4): 1046-1054.
76. Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998 Sep;3(3):155-163.
77. Wrenzycki C, Herrmann D, Lucas-Hahn A, Lemme E, Korsawe K, Niemann H. Gene expression patterns in in vitro-produced and somatic nuclear transfer-derived preimplantation bovine embryos: relationship to the large offspring syndrome. Anim Reprod Sci 2004 Jul;82-83:593-603.
78. Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, et al. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc Natl Acad Sci USA 2001;98(24):13734-13738.
79. Reik W, Walter J. Genomic imprinting: parental influence on the genome. Nat Rev Genet 2001 Jan;2(1):21-32.
80. Reik W, Santos F, Dean W. Mammalian epigenomics: reprogramming the genome for development and therapy. Theriogenology 2003 Jan;59(1):21-32.
81. Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of pre-implantation mouse embryos affects fetal development and the expression of imprinted genes. Biol Reprod 2001;64(3):918-926.
82. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, et al. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001 Feb;27(2):153-154.
83. Niemann H, Wrenzycki C. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. Theriogenology 2000 Jan;53(1):21-34.
84. Wrenzycki C, De Sousa P, Overstrom EW, Duby RT, Herrmann D, Watson AJ, et al. Effects of superovulated heifer diet type and quantity on relative mRNA abundances and pyruvate metabolism in recovered embryos. J Reprod Ferti 2000;118:69-78.
85. Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, et al. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 2002 Sep;67(3):767-775.
86. Niemann H, Wrenzycki C, Lucas-Hahn A, Brambrink T, Kues WA, Carnwath JW. Gene expression patterns in bovine in vitro produced and nuclear transfer-derived embryos and their implications for early development. Cloning Stem Cell 2002; 4(1):29-38.
87. Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K, Niemann H. In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. Biol Reprod 2002 Jan;66(1):127-134.
88. Blelloch R, Wang Z, Meissner A, Pollard S, Smith A, Jaenisch R. Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. Stem Cells 2006 Sep;24(9):2007-2013.
89. Baxter J, Sauer S, Peters A, John R, Williams R, Caparros ML, et al. Histone hypomethylation is an indicator of epigenetic plasticity in quiescent lymphocytes. EMBO J 2004 Nov;23(22): 4462-4472.
90. Selokar NL, Saini M, Palta P, Chauhan MS, Manik R, Singla SK. 2014. Hope for restoration of dead valuable bulls through cloning using donor somatic cells isolated from cryopreserved semen. PLOS One 2014 March 10;9(3):e90755.

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REVIEW ARTICLE

Pathways to Psychiatric Care

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ABSTRACT

It is very important to understand how people seek pathways for psychiatric care especially in a developing nation like India. It is instrumental for planning and organizing psychiatric services for the community. Due to greater prevalence of mental illness in current times, majority of which are from developing nations with limited psychiatric services, studies determining the pathways of psychiatric care need to be undertaken so that mental health services could be planned according to the prevalent cultural norms and other factors more specific to the developing nations like India.

Keywords: Psychiatry, Pathways, Mental health.

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INTRODUCTION

It is very important to understand how people seek pathways for psychiatric care, especially in a developing nation like India. It is instrumental for planning and organizing psychiatric services for the community.[1] Descriptive studies[2,3] regarding this issue demonstrated that people with psychiatric problems follow a variety of pathways before they reach mental health professionals. Their pathways are influenced by various factors which include (a) conventions governing referral, (b) relationships between mental health professionals and other sources of help, (c) the availability and accessibility to mental health facilities and other helping agencies.[1] Fujisawa et al[1] examined pathways to psychiatric care in Japan and found that the patients who consulted mental health professionals as their first care-providers took a longer time before consulting psychiatrists than patients who consulted non-mental health professionals as their first care-providers. They also found that the patients who presented with somatic symptoms as their main problem experienced longer delay from the onset of illness to psychiatric care than the patients who complained about depressive or anxiety symptoms. They concluded that the pathway to psychiatric care in Japan heavily relied on medical resources. Their study emphasized the importance of improving skills and knowledge that would facilitate the recognition of psychiatric disorders in the general health care system.

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PATHWAYS-TO-CARE MODEL

Lincoln and McGorry reviewed the literature about pathways to psychiatric care among young persons experiencing a first episode of psychosis. They concluded that formulation of a pathways-to-care model appears to offer a useful way of understanding mental healthcare use and exploration of consumer experiences would enrich the model. Strategies to reduce treatment delay could then be developed and evaluated. Increased consumer involvement might help ensure that services are better tailored to patients' needs.[4] Gater et al described the referral pathways and documented the factors associated with delays in referral. The pathways in centers relatively well provided with psychiatric staff were dominated by general practitioners and to a lesser extent hospital doctors. The relatively less well resourced centers showed a variety of pathways with native healers often playing an important part. Delays were remarkably short in all centers regardless of psychiatric resources. Somatic problems were a common presentation in all centers and longer delays were found on pathways involving native healers.[2] Anna-Karin et al found that there were important ethnic and social differences in children's and adolescents' pathways to mental healthcare.[5]

FACTORS CAUSING DELAY IN PSYCHIATRIC REFERRAL

Factors causing delay in the initiation of appropriate treatment at the first instance vary from region to region depending upon the sociocultural profile, education, attitude of family/society toward mental illness, perceptions, myths, beliefs, stigma attached with psychiatric disorder, availability/accessibility of psychiatric services and referral patterns, and previous experience of receiving psychiatric help. These determinants also differ in their strength of impact deciding the pathway of care in different geographic regions of world. For example, in the developed nations, the major concern is of stigma, while in the developing nations, it is the problem

of age old cultural myths and supernatural explanations of psychiatric disorders. There is also a significant role of care providers in deciding the pathways to psychiatric care, the first care provider being the most important for giving direction to the pathway of care to seek further help. Trivedi and Jilani emphasized the need for research to delineate pathways to psychiatric care and their determinants in the developing countries like India.[6] Research related to help seeking behavior and attitude toward mental illnesses and services which primarily determine the pathway of care has been carried out mainly in developed nations. There is, however, deficiency of information from the developing countries.[7]

Further studies in the arena of pathways to psychiatric care and its associated factors, such as culture, sources of referral, access to mental health professionals, indigenous systems of healthcare, attitude toward psychiatric disorders, and perceptions of patients and relatives toward psychiatry are needed. Due to greater prevalence of mental illness in current times, majority of which are from developing nations with limited psychiatric services, studies determining the pathways of psychiatric care need to be undertaken so that mental health services could be planned according to the prevalent cultural norms and other factors more specific to the developing nations like India.[8]

REFERENCES

1. Fujisawa D, Hashimoto N, Masamune-Koizumi Y, Otsuka K, Tateno M, Okugawa G, et al. Pathway to psychiatric care in Japan: a multicenter observational study. Int J Ment Health Syst 2008;2:14.
2. Gater R, de Almeida e Sousa B, Barrientos G, Caraveo J, Chandrashekar CR, Dhadphale M, et al. The pathways to psychiatric care: a cross-cultural study. Psychol Med 1991 Aug;21(3):761-774.
3. Gater R, Jordanova V, Maric N, Alikaj V, Bajs M, Cavic T, et al. Pathways to psychiatric care in Eastern Europe. Br J Psychiatry 2005 Jun;186:529-535.
4. Lincoln CV, McGorry P. Who cares? Pathways to psychiatric care for young people experiencing a first episode of psychosis. Psychiatr Serv 1995 Nov;46(11):1166-1171.
5. Anna-Karin I, Svensson R, Adler H, Levander S, Rydelius PA, Levander MT. Pathways to child and adolescent psychiatric clinics: a multilevel study of the significance of ethnicity and neighbourhood social characteristics on source of referral. Child Adolesc Psychiatry Ment Health 2011;5:6.
6. Trivedi JK, Jilani AQ. Pathway of psychiatric care. Ind J Psychiatry 2011 Apr;53(2):97-98.
7. Pradhan SC, Singh MM, Singh RA, Das J, Ram D, Patil B, Jain AK, Thomas JK. First care givers of mentally ill patients: a multicenter study. Ind J Med Sci 2001 Apr;55(4):203-208.
8. Lincoln C, Harrigan S, McGorry PD. Understanding the topography of the early psychosis pathways. An opportunity to reduce delays in treatment. Br J Psychiatry Suppl 1998;172(33): 21-25.

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REVIEW ARTICLE

Stem Cells Cloning and Therapeutic Potential

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ABSTRACT

Stem cells have huge potential to transform current manner in which medicine is practiced. Rather than treating diseased cells with medicines and antibiotics, stem cells can just replace the diseased cells with healthy cells. But it will take time before this research gets translated to the clinic. At present, various types of stem cells like human embryonic stem (hES) cells, induced pluripotent stem (iPS) cells, fetal stem cells, adult tissue-specific stem cells (HSCs, MSCs, etc.), very small embryonic-like stem cells (VSELs) and related technologies like therapeutic cloning are subject to extensive research. Clinicians appear to be in a hurry to apply the stem cells to their patients and there is a huge industry banking stem cells for future autologus use. However, the scientific community is still not sure which is the best stem cell candidate for regenerative medicine. The chapter provides an update on various fronts and also discusses whether there exists a need to bank stem cells for future use. The author is puzzled by realizing as to what needs to be repaired/regenerated—the stem cells or their microenvironment 'niche'!

Keywords: Stem cells, ES cells, iPS cells, SCNT, VSELs, Regeneration.

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INTRODUCTION

Potential of stem cells to regenerate diseased organs has raised hopes of many. Of all the stem cells, human embryonic stem (hES) cells, reported for the first time by Thomson's group[46] have maximum regenerative potential as they are pluripotent in nature and can give rise to all the three germ layers and 200 hundred odd cell types in the body. However, these stem cells have two major associated concerns including (i) immune rejection after cell therapy and (ii) risk of teratoma formation. Embryonic stem cells are derived from the inner cell mass of spare human blastocyst, express HLA antigens when they differentiate into various committed cell types and hence face the issue of immune rejection.

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This immune rejection issue can be overcome by either making patient specific stem cells by cloning or by reprogramming adult somatic cells to embryonic state by using 'Yamanaka factors'.[45] The later approach results in the expansion of induced pluripotent stem (iPS) cells and this technology developed by Prof Shinya Yamanaka was awarded Nobel Prize in Medicine in 2012. Indeed this award was shared with Prof JB Gurdon who had reported 40 years earlier that on replacing the nucleus of a frog egg by a mature cell from the intestine resulted in the development of a normal tadpole.[9] Thus, he showed for the first time that oocyte cytoplasm had factors that could reprogram a somatic cell to embryonic state. This process of reproductive cloning resulted in the birth of first mammal—Dolly, the sheep in 1996 at the Roslin Institute in Scotland after 227 attempts. An udder cell from a 6-year-old sheep was injected in an enucleated unfertilized egg and later transplanted *in utero* resulted in the birth of Dolly which also produced a normal offspring. However, Dolly had to be euthanized at the age of six and half (against a normal age of 11-12 years), because she developed arthritis in her hind leg and lung tumor—both being diseases which manifest with age. Her chromosomal telomeres were also shorter than other sheep of her age. All this implied that she aged early as she was formed from an udder cell of a 6-year-old sheep. A modification of reproductive cloning (which is not permitted in humans) is therapeutic cloning whereby a somatic cell can be injected in an enucleated egg to produce a blastocyst which can then serve as a source of inner cell mass to grow embryonic stem cells (Fig. 1). These ES cells will be patient specific and immune rejection could be overcome. This technology is also termed somatic cell nuclear transfer and falls under the category of restricted area of research based on the Indian Council of Medical Research and Department of Biotechnology (ICMR-DBT) stem cell guidelines.

Stem Cell Biology Department was established at National Institute for Research in Reproductive Health (NIRRH) in 2003 with a focus to derive well-characterized human embryonic stem cell lines on human feeders. We successfully developed two human ES cell lines KIND-1 and KIND-2[17] and have differentiated them into pancreatic and tripotent cardiovascular progenitors to hopefully treat diabetes and cardiac diseases.[20,28,30] An International Training Course with a focus on therapeutic cloning to generate autologus embryonic stem cells was organized in 2007 at

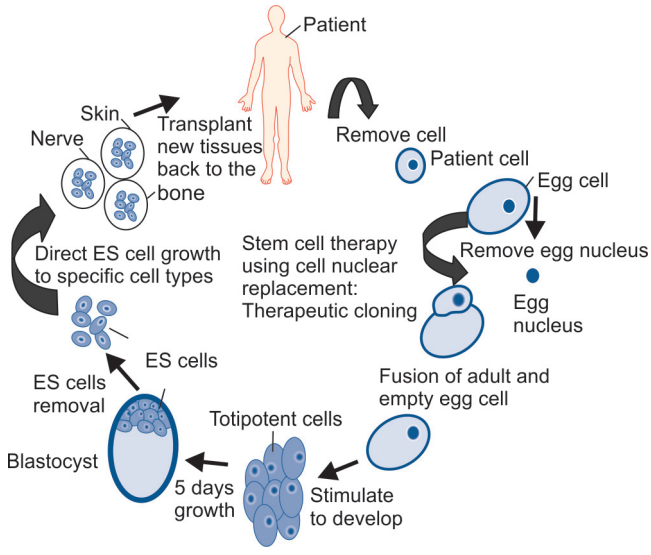

Fig. 1: Concept of somatic cell nuclear transfer to derived patient-specific embryonic stem cells

NIRRH with Dr William Ritchie (embryologist who was involved in making Dolly) from Roslin Institute as one of the Course Directors. Department of Biotechnology, Government of India provided us generous support to establish the technology of somatic cell nuclear transfer (SCNT) using sheep eggs.[21] Human eggs are required for carrying out SCNT, but they are a scarce commodity and using human eggs for SCNT is an extremely sensitive ethical issue, especially after the Hwang episode in Korea.[6]

A project to establish patient specific ES cell lines by SCNT has been submitted by us for various approvals, however, it has been more than 6 to 7 years but the study has not yet been initiated. Meanwhile, our research efforts have identified a novel population of pluripotent stem cells in adult body tissues termed very small embryonic-like stem cells (VSELs). The existence and potential of VSELs make the need to carry out SCNT unnecessary and we also contemplate that when pluripotent stem cells exist in adult body tissues—what is the need of pluripotent ES/iPS cells grown in a Petri dish for regenerative medicine. This chapter provides a broad overview on the advances made using these different kinds of stem cells and related technologies.

CURRENT STATUS OF SCNT

Somatic cell nuclear transfer was described as the method of the year 2013 when Prof Mitalipov from Oregon published the derivation of human ES cell lines by SCNT.[44] It was the most discussed study of 2013 and the efficiency was described as one egg donation cycle will give rise to 8 mature oocytes and hopefully 4 cell lines. The group reported several genetic abnormalities in iPS cells but not in cell lines derived by SCNT.[19,32] He mentioned that it took 6 years to overcome regulatory hurdles but only 3 months to do the work. Future of the technology depends on the policymakers and how various countries regulate egg donors and make them available for research.

CURRENT STATUS OF ES AND IPS CELLS RESEARCH AND THERAPY

Embryonic stem and iPS cells have been differentiated into various lineages and their efficacy and safety has been studied in animal models. Few clinical trials were initiated using ES cells including for spinal cord injury by Geron and macular dystrophy and degeneration by ACT company in Massachusetts using RPE cells derived from ES cells.[38] Similarly, first clinical trial using human iPS cells may be soon initiated in RIKEN Center in Kobe, Japan for retinal degeneration and one proposed by Kapil Bharti will be supported by NIH again targeting age-related macular degeneration of the retina. However, the NIH stem cell program with a major focus on iPS cells has closed down apparently, because NIH did not approve several iPS based clinical trials.[36] So, what was the reason for such a drastic step? A careful review of literature provides us with an answer. The very origin of iPS cells from primary skin fibroblasts culture still remains ambiguous and have many associated reprogramming issues.[7,19,29] Is it really the fibroblast that gets reprogramed or a VSEL present in that culture that starts growing or is there a sub-population of stem cells that start growing? The efficiency of derivation of iPS cells remains extremely poor (<0.05%)! If fibroblasts get reprogramed—all the cells in culture should get reprogramed!

The emerging literature suggests that human ES cells differentiate into their fetal counterparts and thus their potential to regenerate adult organs has become a big question mark.[43] On the same note, ES/iPS cells have been used by several investigations to make gametes, but the aim to derive gametes from ES/iPS stem cells still remains a distant dream.[8,10,14,18,48,49] Recent success was achieved using mouse ES/iPS cells to make gametes but the offspring were genetically affected.[11-13] What is the reason—why despite huge investment of funds, huge research efforts we are still far from taking pluripotent stem cell research from bench to the bed side? It is time to slow down and think and we should be open to mid-course corrections. Besides ES/iPS cells, mesenchymal cells have also been a subject of extensive investigations.[31] Several autologus stem cell trials have been conducted worldwide and a recent interesting update in the field is provided by Nowbar and group.[22] One success story from our country is restoration of vision using limbal stem cells by Balasubramanian's group at LV Prasad Eye Institute, Hyderabad.[37]

INTRODUCTION TO VERY SMALL EMBRYONIC-LIKE STEM CELLS (VSELs)

Besides ES and iPS cells, another type of pluripotent stem cell were the embryonic germ cells reported by Shamblott's group[40] and derived from migrating primordial germ cells (PGCs). These stem cells are pluripotent just like ES cells but do not expand in culture as well.[47] The PGCs migrate along the dorsal mesentery, settle in the gonadal ridge and differentiate into germ cells. Recent literature shows that the PGCs in addition to settling in the gonadal ridge—also migrate an settle in various developing organs—sit on the top of hierarchy of all tissue-specific adult stem cells in the body and survive throughout life as VSELs which act as a backup pool for the adult tissue specific stem cells. These stem cells were described for the first time by Professor Ratajczak's group at the University of Louisville, USA.[15,35] Very small embryonic-like stem cells are very small in size, easily discarded during volume reduction step during processing of cord blood, bone marrow and various other tissues.[3,39] Very small embryonic-like stem cells have been proposed to be similar to late migrating PGCs.[41] Similar to hES cells, VSELs are pluripotent however may prove to be better than human ES/iPS cells in a clinic, because (i) they can be isolated from autologus source and thus immune rejection issues do not exist and (ii) they do not form teratoma. There is no comparison of VSELs with adult stem cells, since it is becoming apparent that indeed VSELs give rise to various adult stem cells. Very small embryonic-like stem cells are easily mobilized in case of injury.[16,23,33,50] We have published extensively on VSELs in the gonads.[4]

Whatever autologus stem cell trials that have been conducted worldwide till date, VSELs have been invariably discarded during processing. Thus, an urgent need exists to undertake clinical studies with these stem cells. The therapy will be absolutely safe, since they will be from an autologus source, but hopefully the efficacy will be higher.

Thus, it is time for stem cell biologists to sit and retrospect. There is a need to brain storm how to move forward in the field of regenerative medicine. Is it necessary to play with emotions of a common man and invite them to bank their teeth stem cells, menstrual blood stem cells, cord blood stem cells etc? Ultimately it is the VSELs that will make all the difference and whatever the source – it is this stem cell that needs to be exploited. Whenever required, they can be easily isolated from the patients' adipose tissue, bone marrow or any other organ. I personally do not see any scope of banking stem cells!

One problematic issue of major cancer is that VSELs exist in very few numbers and they do not expand in culture—then how will they be useful for regeneration. We need to realize that VSELs give rise to progenitors which undergo clonal expansion in large numbers to maintain homeostasis. Thus, very few VSELs are enough for a large number of progenitors. The million dollar question remains as to how we could exploit the VSELs for regenerative medicine and also that despite their natural mobilization—why regeneration does not occur! These are mind boggling questions and we are coming to realize that rather than stem cells—it is the niche which is more crucial and gets compromized with age. If the scientists could repair the niche, stem cell biology will be normalized and regeneration will occur. Our group has reported VSELs in adult human testes,[2] adult mammalian ovaries,[24-27] bone marrow and cord blood.[3] Recently, we have shown that VSELs are capable of regenerating adult mouse pancreas after partial pancreatectomy.[5] We also reported that VSELs survive in the testis[1] as well as in the ovary[41,42] after chemotherapy. Further, we were able to restore testicular function by reconstructing the niche.[1] It is believed that VSELs could be the possible embryonic remnants existing in various adult body organs responsible for various cancers.[34]

To conclude stem cell biology field although having great clinical relevance, is still in infancy and lot more basic research is required and also brainstorming to decide on which stem cells to use and how for achieving regeneration.

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REFERENCES

1. Anand S, Bhartiya D, Sriraman K, Patel H, Manjramkar DD. Very small embryonic-like stem cells survive and restore spermatogenesis after busulphan treatment in mouse testis. Accepted J Stem Cell Res Ther 2014.
2. Bhartiya D, Kasiviswanathan S, Unni SK, Pethe P, Dhabalia JV, Patwardhan S, et al. Newer insights into premeiotic development of germ cells in adult human testis using Oct-4 as a stem cell marker. J Histochem Cytochem 2010;58(12):1093-1106.
3. Bhartiya D, Shaikh A, Nagvenkar P, Kasiviswanathan S, Pethe P, Pawani H, et al. Very small embryonic-like stem cells with maximum regenerative potential get discarded during cord blood banking and bone marrow processing for autologous stem cell therapy. Stem Cells Dev 2012;21(1):1-6.
4. Bhartiya D, Unni S, Parte S, Anand S. Very small embryonic-like stem cells: implications in reproductive biology. Biomed Res Int 2013, 2013:682326.
5. Bhartiya D, Mundekar A, Mahale V, Patel H. Very small Embryonic-like stem cells (VSELs) are involved in regeneration of mouse pancreas post-pancreatectomy. Accepted in J Stem Cell Res The 2014.

6. Check E. Where now for stem-cell cloners? Special Report. Nature 2005;438:1058-1059.
7. Cyranoski D. iPS cells in humans. Nat Biotechnol 2013;31(9):775.
8. Easley CA 4th, Latov DR, Simerly CR, Schatten G. Adult somatic cells to the rescue: nuclear reprogramming and the dispensability of gonadal germ cells. Fertil Steril 2014;101(1):14-19.
9. Gurdon JB. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J Embryol Exp Morphol 1962;10(4):622-640.
10. Hanna CB, Hennebold JD. Ovarian germline stem cells: an unlimited source of oocytes. Fertil Steril 2014;101(1):20-30.
11. Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. Science 2012;338(6109):971-975.
12. Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell 2011;146(4):519-532.
13. Hayashi K, Saitou M. Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. Nat Protoc 2013; 8(8):1513-1524.
14. Hou J, Yang S, Yang H, Liu Y, Liu Y, Hai Y, et al. Generation of male differentiated germ cells from various types of stem cells. Reproduction 2014;147(6):R179-R188.
15. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, et al. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 2006;20(5):857-869.
16. Kucia MJ, Wysoczynski M, Wu W, Zuba-Surma EK, Ratajczak J, Ratajczak MZ. Evidence that very small embryonic-like stem cells are mobilized into peripheral blood. Stem Cells 2008;26(8): 2083-2092.
17. Kumar N, Hinduja I, Nagvenkar P, Pillai L, Zaveri K, Mukadam L, et al. Derivation and characterization of two genetically unique human embryonic stem cell lines on in-house-derived human feeders. Stem Cells Dev 2009;18(3):435-445.
18. Legro RS, Adashi EY. Introduction: germline stem cell therapy in humans: two are not enough. Fertil Steril 2014;101(1):1-2.
19. Ma H, Morey R, O'Neil RC, He Y, Daughtry B, Schultz MD, et al. Abnormalities in human pluripotent cells due to reprogramming mechanisms. Nature 2014;511(7508):177-183.
20. Nagvenkar P, Pethe P, Pawani H, Telang J, Kumar N, Hinduja I, et al. Evaluating differentiation propensity of in-house derived human embryonic stem cell lines KIND-1 and KIND-2. In Vitro Cell Dev Biol Anim 2011;47(5-6):406-419.
21. Nandedkar P, Chohan P, Patwardhan A, Gaikwad S, Bhartiya D. Parthenogenesis and somatic cell nuclear transfer in sheep oocytes using Polscope. Indian J Exp Biol 2009;47(7):550-558.
22. Nowbar AN, Mielewczik M, Karavassilis M, Dehbi HM, Shun-Shin MJ, Jones S, Howard JP, Cole GD, Francis DP. Discrepancies in autologous bone marrow stem cell trials and enhancement of ejection fraction (DAMASCENE): weighted regression and meta-analysis. BMJ 2014 Apr 28;348:g2688.
23. Paczkowska E, Kucia M, Koziarska D, Halasa M, Safranow K, Masiuk M, et al. Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. Stroke 2009;40(4):1237-1244.
24. Parte S, Bhartiya D, Manjramkar DD, Chauhan A, Joshi A. Stimulation of ovarian stem cells by follicle stimulating hormone and basic fibroblast growth factor during cortical tissue culture. J Ovarian Res 2013;6(1):20.
25. Parte S, Bhartiya D, Patel H, Daithankar V, Chauhan A, Zaveri K, et al. Dynamics associated with spontaneous differentiation of ovarian stem cells in vitro. J Ovarian Res 2014;7(1):25.
26. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K, et al. Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary. Stem Cells Dev 2011;20(8):1451-1464.
27. Patel H, Bhartiya D, Parte S, Gunjal P, Yedurkar S, Bhatt M. Follicle stimulating hormone modulates ovarian stem cells through alternately spliced receptor variant FSH-R3. J Ovarian Res 2013;6:52.
28. Pawani H, Nagvenkar P, Pethe P, Bhartiya D. Differentiation of human ES cell line KIND-2 to yield tripotent cardiovascular progenitors. In Vitro Cell Dev Biol Anim 2013;49(1):82-93.
29. Pera MF. Stem cells: the dark side of induced pluripotency. Nature 2011;471(7336):46-47.
30. Pethe P, Nagvenkar P, Bhartiya D. Polycomb group protein expression during differentiation of human embryonic stem cells into pancreatic lineage in vitro. BMC Cell Biol 2014;15:18.
31. Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views. Stem Cells 2007;25(11): 2896-2902.
32. PolejaevaI, Mitalipov S. Stem cell potency and the ability to contribute to chimeric organisms. Reproduction 2013;145(3): R81-88.
33. Ratajczak MZ, Kucia M, Shin DM, Rui L, Drukala J, Marlicz W, et al. A unique population of mobile very small embryonic/epiblast like (VSEL) stem cells resides in adult tissues: physiological and pathological consequences. Cell Ther Transplant 2008;1:36-43.
34. Ratajczak MZ, Shin DM, Liu R, Marlicz W, Tarnowski M, Ratajczak J, Kucia M. Epiblast/germ line hypothesis of cancer development revisited: lesson from the presence of Oct-4+ cells in adult tissues. Stem Cell Rev 2010;6(2):307-316.
35. Ratajczak MZ, Zuba-Surma E, Wojakowski W, Suszynska M, Mierzejewska K, Liu R, Ratajczak J, Shin DM, Kucia M. Very small embryonic-like stem cells (VSELs) represent a real challenge in stem cell biology: recent pros and cons in the midst of a lively debate. Leukemia 2014;28(3):473-484.
36. Reardon S. NIH stem-cell programme closes. Nature 2014; 508(7495):157.
37. Sangwan VS, Basu S, Vemuganti GK, Sejpal K, Subramaniam SV, Bandyopadhyay S, Krishnaiah S, Gaddipati S, Tiwari S, Balasubramanian D. Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. Br J Ophthalmol 2011 Nov;95(11):1525-1529.
38. Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, et al. Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 2012;379(9817): 713-720.
39. Shaikh A, Bhartiya D. Pluripotent stem cells in bone marrow and cord blood. In: 'Blood Cells—An Overview of Studies in Hematology', Publisher—InTech, Europe, Dr Terry Moschandreou, editor. 2012:69-88.
40. Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, et al. Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci USA 1998;95(23):13726-13731.
41. Shin DM, Liu R, Klich I, Ratajczak J, Kucia M, Ratajczak MZ. Molecular characterization of isolated from murine adult tissues very small embryonic/epiblast like stem cells (VSELs). Mol Cells 2010;29(6):533-538.

42. Sriraman K, Bhartiya D, Anand S, Bhutda S. Mouse ovarian very small embryonic-like stem cells resist chemotherapy and initiate oocyte-specific differentiation in vitro in response to follicle stimulating hormone. Under Review in Reproductive Sciences 2014.
43. Tabar V, Studer L. Pluripotent stem cells in regenerative medicine: challenges and recent progress. Nat Rev Genet 2014;15(2):82-92.
44. Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, et al. Human embryonic stem cells derived by somatic cell nuclear transfer. Cell 2013;153(6):1228-1238.
45. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126(4):663-676.
46. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282(5391): 1145-1147.
47. Turnpenny L, Brickwood S, Spalluto CM, Piper K, Cameron IT, Wilson DI, et al. Derivation of human embryonic germ cells: an alternative source of pluripotent stem cells. Stem Cells 2003; 21(5):598-609.
48. Valli H, Phillips BT, Shetty G, Byrne JA, Clark AT, Meistrich ML, et al. Germline stem cells: toward the regeneration of spermatogenesis. Fertil Steril 2014;101(1):3-13.
49. Volarevic V, Bojic S, Nurkovic J, Volarevic A, Ljujic B, Arsenijevic N, et al. Stem cells as new agents for the treatment of infertility: current and future perspectives and challenges. Biomed Res Int 2014; 2014: 507234.
50. Zuba-Surma EK, Kucia M, Dawn B, Guo Y, Ratajczak MZ, Bolli R. Bone marrow-derived pluripotent very small embryonic-like stem cells (VSELs) are mobilized after acute myocardial infarction. J Mol Cell Cardiol 2008;44(5):865-873.

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REVIEW ARTICLE

Regulation of Growth and Function of Lifeline Placenta

A Jagannadha Rao

ABSTRACT

Placenta is an association of fetal and maternal tissues which develops during pregnancy. Placenta is often called lifeline, because it is the link between mother and growing fetus. It serves variety of functions, which include transport of nutrients to growing fetus, waste products from fetus, exchange of gases and also immunological protection to the fetus. It has a unique ability to function as a hypothalamo-pituitary-gonadal-axis as it can produce a variety of peptide, protein and steroid hormones. Thus, it is an autonomous unit capable of regulating its own growth and function.

Keywords: Placenta, Cytotrophoblast, Syncytiotrophoblast, hCG, Differentiation, Telomerase.

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INTRODUCTION

Placenta is an association of fetal and maternal tissues which develops during pregnancy and is often called lifeline, because it is the link between mother and growing fetus. Placenta serves variety of functions, which include transport of nutrients to growing fetus, waste products from fetus, exchange of gases, providing immunological protection as the fetus is an allograft. The proper development and growth of placenta is of utmost importance and critical in the outcome of successful pregnancy. In fact, now it is accepted that several adult diseases have fetal origin and maternal health influences the outcome of pregnancy and well being of fetus and new born. Poor nutrition, anemia, infections, etc. during pregnancy affect not only the health of pregnancy woman, but also the growth of the fetus. Several pregnancy related disorders, for example, Intra uterine growth retardation (IUGR), Pre-eclampsia, Spina bifida, etc. have their origin in nutritional deficiencies during pregnancy. Thus, the key link between the mother and fetus is placenta by providing adequate nutrition and other support such as providing oxygen, removal of waste products and hormonal support for growth and metabolism to the fetus.[1]

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REGULATION OF GROWTH OF PLACENTA

It is interesting to note that placental growth occurs only during early pregnancy. Subsequently it grows only in size. It has been established that placental weight is related to the weight of the new born. Several factors influence the growth and function of placenta among which are nutritional status of mother, level of estrogen, progesterone, gonadotropin releasing hormone (GnRH) and several other growth factors. Earlier studies from our laboratory have shown that estrogen plays an important role in regulation of growth and function of placenta in humans. We have shown that estrogen stimulates general as well as specific protein synthesis in placenta.[2]

Considering the importance of the placenta in pregnancy, we have studied the regulation of growth of placenta using the molecular marker of growth namely Telomerase (TR). TR is a Ribonucleoprotein enzyme complex and functions to compensate for replication-associated loss in telomeric repeats. Its activity correlated with cell immortalization. Most human tumors are TR[1] positive whereas somatic tissues are TR negative. In the human fetus, TR activity is detectable in many tissues early in gestation. During development, there is tissue-specific loss in TR activity. Human placenta is similar to many TR positive tumors and malignant trophoblastic diseases exhibit very high telomerase activity in the context of placenta. The normal villi have lower levels of TR activity and villi from cases of IUGR/pre-eclampsia show undetectable levels. Placenta resembles a tumor in terms of it invasive characteristics but unlike tumors, it is a well controlled invasion in placenta.[3]

Placenta serves as a transient hypothalamo-pituitary axis by its ability to produce, proteins, peptides and steroid hormones. Of the several hormones produced by the placenta steroid such as estrogen and progesterone are produced relatively in large quantities, during pregnancy.[4] Of these two hormones namely, estrogen and progesterone, estrogen is a known mitogenic hormone and with the progress in pregnancy there is a rapid increase in the serum and placental levels of estrogens. This coincides with differentiation of cytotrophoblasts into syncytiotrophoblasts. Our studies revealed that estrogen induces differentiation of cytotro-

phoblasts in to syncytiotrophoblasts and interestingly as mentioned earlier there is significant increase in estrogen production during the differentiation process. Differentiation process which could be induced by estrogen could be blocked by addition of ICI182780, an estrogen receptor antagonist. It is known that cytotrophoblasts are highly proliferative and high in telomerase activity which is an indicator of growth. As mentioned earlier TR activity decreases during differentiation of cytotrophoblasts into syncytiotrophoblasts. In an attempt to elucidate the mediator of the estrogen indeed differentiation of cytotrophoblasts into syncytiotrophoblasts. We analyzed the levels of TGFβ1 known for its role in cellular differentiation. Interestingly there was a significant increase in the expression of TGFβ1 both at the mRNA levels as assessed by RT-PCR, as well as at protein level as assessed by Western blot analysis. In an attempt to establish the role of TGFβ1, we have further investigated the effect of addition of TFGβ1 on the TR activity. Interestingly, there was significant decrease in the TR activity along with differentiation of the cytotrophoblasts into syncytiotrophoblasts following addition of TGFβ1.[5-8]

REGULATION OF ENDOCRINE FUNCTION OF PLACENTA

As mentioned earlier the human placenta also serves as a very efficient endocrine gland by its ability to synthesize and secrete a variety of protein, peptide and steroid hormones. In most of the primate species, not only the steroid hormone producing function of ovary but also the function of hypothalamo-pituitary axis is taken over by the placenta. Human placenta produces protein hormones like chorionic gonadotropin (CG) and chorionic somatomammotropin similar to luteinizing hormone (LH) and growth hormone (GH) respectively, produced by the pituitary. The large quantities of progesterone and estrogens, which are produced by the ovary, are also produced by placenta. It has also been demonstrated that several peptides like GnRH adrenocortico trophic hormone (ACTH) and relaxin, etc. are produced the placenta.[9] Considering this, it is justified in suggesting that human placenta serves as a transient hypothalamo-pituitary-gonadal axis during pregnancy. Although the regulatory interrelationship between hypothalmo-pituitary gondal axis has not been established in placenta. However, it is likely that the regulatory mechanism seen in the hypothalamo-pituitary-gonadal axis could be operative in the placenta also.[4]

It has been well established that pituitary LH is under the control of hypothalamic GnRH. Considering the fact that CG is very similar to LH both structurally and functionally, it is likely that CG may also be regulated by placental GnRH. Our investigations demonstrating the presence of specific receptors for GnRH as demonstrated by the binding of ^{125}1 GnRH to human placental cell membranes support this assumption. The presence of GnRH transcripts in human placenta has been demonstrated by northern analysis of the human placenta RNA.[10,11]

Although distinct signal corresponding to 1.8 kB was seen in human first trimester human placenta, the levels of GnRH transcript appears to be low in the term placenta. Additional evidence was provided by demonstrating the presence of GnRH receptor protein in the placental membranes probed using a specific antiserum raised to the bonnet monkey pituitary GnRH receptor fragment. A signal corresponding to approximate 70 kDa which corresponded to the size of rat pituitary GnRH receptor was observed.[12] Following this we examined *in vitro* effect of the addition of GnRH on CG levels. Increase in both immunoreactive CG level as well as synthesis of CG was noticed. Further inclusion of specific GnRH receptor antagonist prevented increase in the levels of CG. Furthermore, addition of Busserelin, an analog of GnRH resulted in an increase in immunoreactive mCG in the monkey placenta. Additional evidence to support the conclusion that GnRH stimulates the secretion of CG in human placenta was provided by studies indicating the involvement of Ca_2^{++} in mediating the GnRH stimulated CG secretion.[13,14] The involvement of GnRH in regulation of CG under *in vivo* conditions was provided by demonstrating that acute or chronic administration of GnRH or Buserelin during early pregnancy in the bonnet monkey resulted in an increase in tile serum level of mCG. Also injection of antiserum GnRH caused a transient but immediate decrease in serum mCG levels.[15]

To investigate the role of P4 and E2 in the regulation of CG synthesis, specific antagonists and inhibitors such as RU 486 and or Progesterone or aromatase inhibitor or tamoxifen (TMX) were added to both first trimester human placenta (FTHP) and term placental (TP) minces under *in vitro* conditions. It was found that while the secretion of CG is under negative modulation by E2 us judged by an increase in immunoreactive CG levels as well as mRNA levels specific for alpha and beta CG subunits, addition of RU486 or dexamethasone or P4 resulted in an increase in the levels of immunoreactive CG protein and transcripts. It was interesting to note that the addition of dexamethasone had differential effect, in that while a combination of RU486 and dexamethasone had an additive effect on CG mRNA levels, in FTHP no such effect was seen on hCG mRNA level. The negative modulation of synthesis of CG by E2 was seen even in the monkey placenta where in, addition of

TMX resulted in an increase in immunoreactive CG level. Although, both TMX and RU486 are known to be estrogen and PR antagonists respectively, we believe they are acting as agonists in the human placenta. It is well known that these compounds can exert both agonistic and antagonist effects depending on the concentration, species, tissue and gene in question.[16-18]

Very little information is available on the possible factors involved in the regulation of P4 production by placenta. The capacity of placenta to synthesize cholesterol *de novo* from acetate is limited and all the cholesterol needed for P4 synthesis is obtained from maternal circulation in the form of low density lipoprotein (LDL) which is taken up by specific receptors on the placental membranes. The uptake of LDL by specific receptors is rate limiting in the biosynthesis of P4 in placenta. Also one of the earliest steps in the stimulation of adrenal steroids by ACTH or gonadal steroids by gonadotropin is up regulation of LDL receptor expression. Considering this, examined the effect of addition of hCG on LDL receptor expression in human placenta. Our results indicated that hCG up regulates LDL receptor expression in human placenta. In addition it was found that both E2 and P4 have a role in regulation of LDL receptor expression.[19]

Although the human placenta is a discarded tissue at the end of pregnancy it serves as an excellent model to investigate a variety of physiological and molecular mechanisms operating in the placenta. As mentioned earlier, it is an excellent model to study the proliferation mechanisms operating in cancer cells. The endocrine function of the human placenta is unique in that it resembles the hypothalamo pituitary axis. However, it should be noted that no neural mechanisms operate in the regulation of synthesis and secretion of variety of hormones. The human placental system consisting of cyto and syncytiotrophoblasts is an excellent model to investigate the autocrine and paracrine mechanism of regulation. It need not be emphasized that the fetus being an allograft the placenta is a unique tissue in selectively preventing the rejection of the growing fetus. Selective permeability is a unique property of human placenta and thus it is very evident that the human placental system can be employed as an excellent model to study regulation of transport, growth and differentiation.

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REFERENCES

1. Shanker YG, Rao AJ. Endocrine functions of the human placenta: lessons from the life line. Proceedings of the Indian National Science Academy—Part B: Biological Sciences 1998;B64(1): 35-50.
2. Sharma SC, Kumari U, Dighe RR, Rao AJ. Regulation of protein synthesis in the first trimester human placenta by 17 beta-oestradiol and progesterone. Placenta 1990 Jan-Feb;11(1): 63-74.
3. Rama S, Suresh Y, Rao AJ. TGF beta1 induces multiple independent signals to regulate human trophoblastic differentiation: mechanistic insights. Mol Cell Endocrinol 2003 Aug 29;206 (1-2):123-136.
4. Santra S, Shanker YG, Rao AJ. Regulation of endocrine function in human placenta. Is it similar to hypothalamo-pituitary-gonadal axis? In: Reproductive immunology. Gupta SK, editor. London, Narosa Publishing House 1999. p. 187-197.
5. Rama S, Suresh Y, Rao AJ. Regulation of telomerase during human placental differentiation: a role for TGF beta1. Mol Cell Endocrinol 2001 Sep;182(2):233-248.
6. Yashwanth R, Rama S, Anbalagan M, Rao AJ. Role of estrogen in regulation of cellular differentiation: a study using human placental and rat Leydig cells. Mol Cell Endocrinol 2006 Feb 26;246(1-2):114-120.
7. Rama S, Petrusz P, Rao AJ. Hormonal regulation of human trophoblast differentiation: a possible role for 17-β- estradiol and GnRH. Mol Cell Endocrinol 2004 Apr 15;218(1-2):79-94.
8. Rama S, Rao AJ. Trophoblast 'pseudo-tumorigenesis': significance, and the contributory factors. Reprod Biol Endocrinol 2004 Mar 25;2:15.
9. Shanker YG, Rao AJ. Endocrine functions of the human placenta: Lessons from the life line. PINSA 1998; B64, p. 35-50.
10. Rao AJ, Mathialagan N. A possible role for gonadotropin releasing hormone in regulation of chorionic gonadotropin secretion by primate placenta. Proceedings of Endocrine Society of India, Hyderabad, 1986. p. 4149.
11. Rao AJ. Studies on the chemistry and physiology of primate placental gonadotropin releasing hormone. In: neuroendocrine regulation in fertility control. Vinod Singh, Muralidhar K, editors. Shillong, NEHU Publications 1989. p. 31-39.
12. Sumana S, Rao VS, Shanker YG, Rao AJ. Cloning and characterization of bonnet monkey GnRH receptor. Mol Hum Reprod 2000 May;6(5):415-421.
13. Sharma CS, Rao AJ. Role of calcium in secretion of chorionic gonadotropin by first trimester human placenta. Indian J Exp Biol 1992 Nov;30(11):1105-1110.
14. Sharma CS, Rao AJ. Effect of calcium ion channel antagonists on chorionic gonadotropin secretion. Biochem Mol Biol Int 1997 Dec;43(5):1101-1106.

15. Rao AJ, Chakraborti R. Effect of acute and chronic administration of gonadotropin releasing hormone agonist Buserelin, on serum chorionic gonadotropin, oestradiol-17β and progesterone during early pregnancy in the south Indian bonnet monkey (Macaca radiata). Anim Reprod Sci 1990;22(3): 261-269.
16. Sharma SC, Purohit P, Rao AJ. Role of oestradiol 17β in the regulation of synthesis and secretion of human chorionic gonadotrophin by first trimester human placenta. J Mol Endocrinol 1993 Aug; 11(1):91-101.
17. Shanker YG, Sharma SC, Rao AJ. Expression of progesterone receptor mRNA in the first trimester human placenta. Biochem Mol Biol Int 1997 Sep;42(6):1235-1240.
18. Shanker YG, Rao AJ. Regulation of progesterone biosynthesis in the human placenta by estradiol 17β and progesterone. Biochem Mol Biol Int 1997 Oct;43(3):591-599.
19. Shanker YG, Shetty UP, Rao AJ. Regulation of low density lipoprotein receptor mRNA levels by estradiol 17β and chorionic gonadotropin in human placenta. Mol Cell Biochem 1998 Oct;187(1-2):133-139.

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CASE REPORT

Anterior Circulation Stroke Following Snakebite: A Rare Presentation

[1]Ciji Sebastine, [2]Neha Athale, [3]Jaishree Ghanekar, [4]Sainath Hegde

ABSTRACT

India is estimated to have the highest snakebite mortality in the world. Most fatalities are due to delay in getting the definitive treatment. Most snakebites are inflicted on the lower limbs of farmers, plantation workers, herdsmen, and hunters in rural areas. The viper is one of India's most commonly encountered poisonous snakes and envenomation following viper bite usually leads to consumption coagulopathy. Clinical characteristics include cellulites, renal failure, hemorrhagic manifestations including pituitary and intracranial hemorrhage. In the setting of viper envenomation, large-vessel thrombosis is a very rare occurrence. Also, bilateral anterior cerebral artery infarction, when unrelated to anatomical abnormalities, surgery or trauma, itself is an exceedingly rare event. The following case is an unusual one of bilateral cerebral infarction in ACA territory in an otherwise healthy individual.

Abbreviations: ACA: Anterior cerebral artery; ASV: Anti snake venom; CNS: Central nervous system.

Keywords: Stroke, Snakebite, MRI study, Anterior circulation.

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CLINICAL HISTORY

The case was a 48-year-old female in an otherwise healthy condition, presented with alleged history of snakebite two days earlier. Bite was on third finger of left hand associated with history of swelling of left upper limb. There was no diplopia, slurring of speech or respiratory embarrassment or any signs of bleeding manifestations and seizure episodes. Following the bite patient had lost consciousness and was taken to nearby hospital where she received ASV. She recovered consciousness after 2 hours in hospital but the patient had problem in recognizing people. She complained of headache on waking up and had one vomiting episode. By evening the next day, the patient who was conscious oriented by now realized that she could not move the left side of body. The weakness started with involvement of lower limb and gradually involved upper limb over period of few hours and then the patient was transferred to our hospital. There was no history of incontinence of urine.

ON EXAMINATION

The patient was conscious, oriented in time, place and person, with pulse rate of 88/minute regular, good volume. All peripheral pulses present. BP was 130/90 mm in right upper arm in supine position. On local examination there was swelling of left upper limb till arm. Central nervous system examination revealed fully conscious patient with normal speech. All cranial nerves were normal on examination. Motor system examination showed power of grade 2/5 in left upper limb and grade 1/5 in left lower limb with exaggerated tendon reflexes and extensor plantar response on left side. Patient's Magnetic Resonance Imaging (MRI) was done which revealed bilateral high frontal lobe infarcts and left parahippocampal gyrus (Figs 1A and B). Magnetic Resonance Imaging angiography could not be done due to cost factor.

Routine blood investigations did not reveal any significant abnormality (Table 1).

Examination of fundus did not show any abnormality. ECG and 2D Echo were normal. Patient was treated with tapering doses of Anti Snake Venom, Anticoagulants, Antiplatelets and Low Molecular Weight Heparin. Glycerine $MgSO_4$ dressing of left upper limb was done. Gradually power on left upper limb and lower limb improved and swelling disappeared over a period of 1 week. Patient was discharged after 10 days with regular follow-up and physiotherapy.

Table 1: Blood investigations

Parameters	*Day 1*	*Day 3*
Hemoglobin (gm/dl)	11.6 gm/dl	10.5 gm/dl
WBC/mm^3	12,800/mm^3	11,500/mm^3
Platelets/mm^3	2.0 lakhs	2.2 lakhs
Bleeding time	2 mins 15 secs	2 mins 10 secs
Clotting time	3 mins 45 secs	3 mins 30 secs
Prothrombin time	14 secs	12 secs
Serum creatinine (mg%)	1.6 mg%	0.88 mg%
Sodium (mmol/l)	132 mmol/l	140 mmol/l
Potassium (mmol/l)	2.7 mmol/l	3.2 mmol/l

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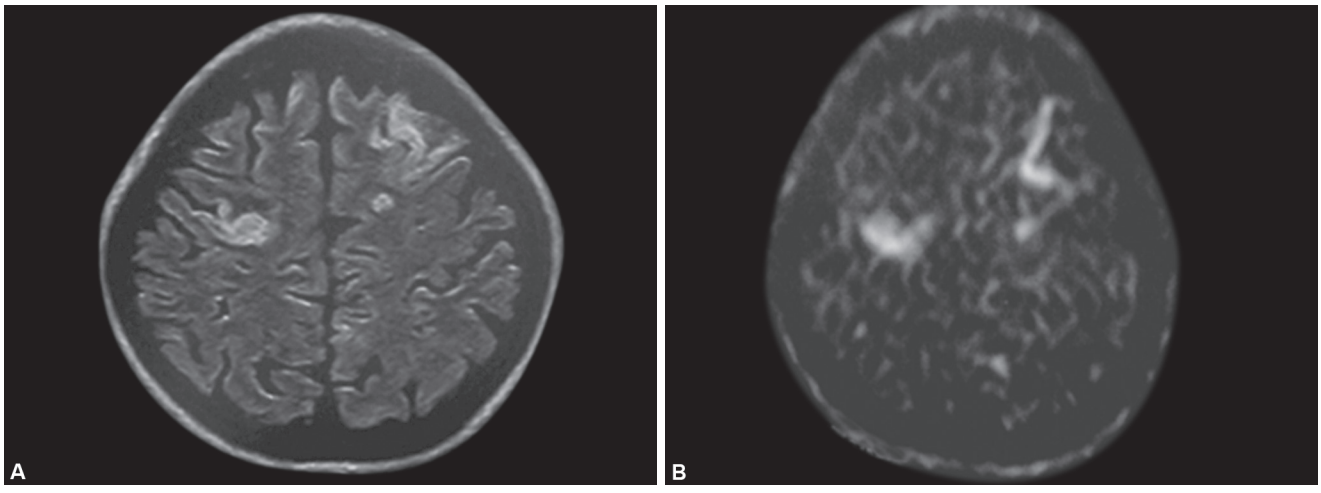

Figs 1A and B: (A) Bilateral high frontal lobe infarcts (T1 image) and (B) left parahippocampal gyrus infarcts

DISCUSSION

In India, more than 2000000 snakebites are reported annually. The most important species are cobras, common krait and Russell's viper. An annual snakebite mortality of 30,000 has been recorded.[1]

Most snakebites are inflicted on the lower limbs of farmers, plantation workers, herdsmen, and hunters in rural areas. The snake is usually trodden at night or in undergrowth. Some enter human dwellings at night and may bite people who roll over on to them while sleeping on the floor. Seasonal peaks in the incidence of snakebite are associated with agricultural activities, such as ploughing before the annual rains. Severe flooding, by concentrating the human and snake populations, has given rise to epidemics of snakebite. Invasion of virgin jungle during construction of new highways and irrigation has led to an increased incidence of snakebite.[2]

Snakebite can either be vasculotoxic or neurotoxic. Vasculotoxic snakebite usually lead to disseminated intravascular coagulation with consumption of clotting factors. Neurological deficits of vasculotoxic snakebite are usually due to intracranial bleeding rather than infarction.

Cerebral complications, particularly ischemic infarction after viper bite is rare[3] Ischemic stroke due to infarction in middle cerebral artery territory following viper bite has been reported by few authors.[4] Brainstem stroke had been reported following Korean viper bite and envenomation from Bathrops lanceolatus that is found only in Matinique.[5] Additionally, the anterior cerebral arteries are infrequent sites of thrombosis, and bilateral infarction in their territories is an exceedingly rare entity.[6]

Vasculotoxic venom is a mixture of numerous enzymes which have opposing effects. One set of enzymes cause hypofibrinogenemia, hypoprothombinemia, thrombocytopenia leading to bleeding manifestations. Other enzymes such as potent proteases lead to activation of clotting factors X and V promoting coagulation. Effect of hyaluronidase causes damage to connective tissue leading to enhanced toxin dissemination.

There is activation of intrinsic coagulation pathway leading to formation of numerous new thrombi in circulation which in turn leads to consumption of coagulation factors and platelets which may result in internal and external bleeding. Another cause is endothelial injury caused by toxic agents such as hemorrhagins which can lead to hemorrhage.[7] Occlusions of arteries due to microthrombi are rare clinical findings.[8]

There are various theories which could lead to ACA territory bilateral infarct.

1. DIC induced by some snake toxins such as viper, cause vessel occlusive thrombi with an underlying procoagulant state which leads to formation of thrombi.[8,9]
2. Variations in viper venom composition, in terms of its hemorrhagic, anticoagulant, and other activities, may favor thrombosis, as opposed to bleeding.[9]
3. Hemorrhagins induces vasospasm in arterioles leads to vasodilatation leading to endothelial damage and increased vascular permeability.
4. Preexisting procoagulant state like mutation in factor V, Protein S and C deficiencies can be another reason. Hyperviscosity state due to hemoconcentration can also be one of the reasons.[10]
5. Hypotension due to hypovolemia from sweating, vomiting, low blood flow area leads to water shed infarcts.

CONCLUSION

We report this case to highlight this uncommon presentation of viper bite (as hemiplegia), with bilateral infarcts in territory of anterior cerebral artery. Our patient, despite treatment with ASV within 1 hour of envenomation developed delayed cerebral infarction on the second day. Clotting time was normal which ruled out coagulopathy as a cause. The

possible cause of infarct in the anterior circulation is due to toxic vasculitis caused by injury to the endothelium by snake venom toxin. Our case also illustrates that one should work up for possible cerebral infarction in a victim of viper envenomation and focal deficit.

REFERENCES

1. David AW, Guideline of clinical management of snakebite for South-East Asia regions. New Delhi: WHO Regional Office for South East Asia, 2005. p. 67.
2. Warrell DA, Cox TM, Firth JD. Oxford Textbook of Medicine. 5th ed. Oxford: Oxford University Press, 2010;1:1329-1340.
3. Bashir R, Jinkins J. Cerebral infarction in a young female following snakebite. Stroke 1985 Mar-Apr;16(2):328-330.
4. Narang SK, Paleti S, Azeez Asad MA, Samina T. Acute is chemic infarct in the middle cerebral artery territory following a Russell's viper bite. Neurol India 2009 Jul-Aug;57(4):479-480.
5. Lee BC, Hwang SH, Bae JC, Kwon SB. Brainstem infarction following Korean viper bite. Neurology 2001 May;56(9): 1244-1245.
6. Minagar A, David NJ. Bilateral infarction in the territory of the anterior cerebral arteries. Neurology 1999 Mar 10;52(4):886-888.
7. Marsh NA. Snake venoms affecting the haemostatic mechanism: a consideration of their mechanisms, practical applications and biological significance. Blood Coagul Fibrinolysis 1994 Jun; 5(3):399-410.
8. Murthy JM, Kishore LT, Naidu KS. Cerebral infarction after envenomation by viper. J Comput Assist Tomogr 1997 Jan-Feb; 21(1):35-37.
9. Prasad NB, Uma B, Bhatt SK, Gowda VT. Comparative characterization of Russell's viper (Daboia/Vipera russelli) venoms from different regions of the Indian peninsula. Biochim Biophys Acta 1999 Aug 5;1428(2-3):121-136.
10. Panicker JN, Madhusudhan S. Cerebral infarction in a young male following viper envenomation. J Assoc Physicians India 2000 Jul;48(7):744-745.

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