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RESEARCH ARTICLE

EFFECT OF MELANOPSIN-SAPORIN CONJUGATE ON MELANOPSIN RETINAL GANGLION CELLS IN MICE

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SUMMARY This study is aimed to selectively ablate melanopsin retinal ganglion cells (mRGCs) subtypes M1 and non-M1 in mice using Melanopsin-Saporin (Mel-SAP) conjugate and examine the specificity of the immunotoxin in DAPI-stained cells of the ganglionic cell layer. Increasing Mel-SAP conjugate concentration from 600 ng/eye to 800 ng/eye led to a drastic reduction in the total population of mRGCs, resulting in a 90% loss of melanopsin cells in a retinal quadrant. M1 and M2 cells showed a proportionate loss of melanopsin cells with increasing concentration of Mel-SAP conjugate application, suggesting that the percentage of cell ablation is dependent on the concentration of Mel-SAP treatment. The Mel-SAP conjugate is not specific to mRGCs alone but affects several other types of ganglion cells. These findings suggest the need for improved specificity of the drug for future studies on nonvisual response processes.

Keywords: Mice, melanopsin-saporin conjugate, melanopsin retinal ganglion cells, circadian rhythm.

INTRODUCTION

In mammals, the eye plays a crucial role as the primary mediator of light input to the central nervous system. Initially, it was believed that only 2 types of photoreceptors, rods and cones, covered visual and nonvisual responses. However, the discovery of the third photoreceptor class, the melanopsin retinal ganglion cells (mRGCs), changed this perspective significantly (Provencio et al. 1998). The retinal ganglion cells (RGCs) are located in the ganglion cell layer near the inner surface of the retina and receive visual information from photoreceptors through 2 intermediate neuron types: bipolar cells and amacrine cells (Belenky et al. 2003). mRGCs are a subtype of RGCs that transmit image forming and nonimage forming visual information from the retina to various regions of the brain (Allen et al. 2019, Yamakawa et al. 2019).

The mammalian eye extracts two fundamentally different types of information from the light environment. Apart from providing a spatial map of the visual scene, it encodes irradiance, which is the ambient level of light. This irradiance information plays a crucial role in several nonimage forming behavioural and physiological responses to light, such as photo entrainment of the circadian rhythm (Lucas et al. 2020), constriction of the pupil, acute suppression of pineal melatonin, acute suppression of activity (masking) in nocturnal mammals, and regulation of sleep latency (Ecker et al. 2010, Reifler et al. 2015).

mRGCs are a specialized class of RGCs that directly respond to light and encode ambient light (irradiance) for nonimage forming visual functions (Hatori et al. 2008). These cells receive signals from rods and cones and are classified into 5 subtypes, M1 to M5, based on their soma size, response sensitivity and dendritic stratification. The M1 cells stratify in the OFF sublayer of the inner plexiform layer (IPL), M2 cells in the ON sublayer, while M3 cells stratify in both (Berson et al. 2010). In a normal retina, M1 cells constitute approximately 40–45%, while non-M1 cells constitute 45–50% of mRGCs (Baver et al. 2008). Moreover, M1 cells have a smaller soma and a simpler dendritic arbor but higher melanopsin levels, resulting in higher sensitivity and faster light response kinetics than the other melanopsin cells (Berson et al. 2010). However, the specific contribution of these subtypes of melanopsin cells in nonimage forming functions remains unknown.

In this study, we explore a potential method for selective ablation of mRGC subtypes, using melanopsin-saporin conjugate (Mel-SAP), to understand the functional role of specific mRGC subtypes. The immunotoxin Mel-SAP is a conjugate of melanopsin antibody and a ribosome inactivating protein, saporin, and is known to specifically ablate mRGCs in a fully differentiated adult retina (Göz et al. 2008). The effect of Mel-SAP is dose-dependent, but even at very high concentration, it does not kill all mRGCs, raising the possibility that its effect is specific to certain mRGC subtypes.

MATERIAL AND METHODS

Adult mice (C57BL/6J, 8–12 wks old, male) were obtained from the animal house, approved by the Institutional Animal Ethics Committee of the National Brain Research Centre, India. They were maintained on a 12 h light:dark cycle with an average ambient daylight of approximately 200 lux.

Bovine serum albumin (BSA), Triton-X100, Na_2HPO_4, NaH_2PO_4, NaCl, KCl and paraformaldehyde were procured from Sigma chemicals, St. Louis (USA). Mel-SAP was obtained from Applied Targeting Systems, San Diego (USA). Alexa Flour-488 from Molecular Probes and DAPI from Vectasheilds, Oregon (USA) were procured for fluorescence labelling. Donkey serum and nitrocellulose membrane were purchased from Millipore (USA). Ketamine (Neon Laboratories Ltd. Thane, India) and Xylazine (Indian Immu-nologicals Ltd. Hyderabad, India) were used as anesthetic agents.

Immunotoxin injection and whole mount retina preparation

Adult mice (C57BL/6J, 8–12 wks old) were injected intravitreally 100, 200, 400, 600 or 800 ng of Mel-SAP conjugate in 2 ul of PBS per eye. Retinas were removed 4 d later from the anesthetized animal (ketamine [100 mg/kg] + xylazine [10 mg/kg] was given for anesthetizing). A small incision was made perpendicular to limbus and immersed in 4% paraformaldehyde (PFA; pH 7.4) at 4° C for 10 min and hemisected, followed by fixation of the posterior eye cup in 4% PFA for 45–60 min at 4° C (Berson et al. 2010). The fixed retina was divided into 4 quadrants, and one of the quadrants was considered for immunohistochemistry.

Immunohistochemistry

One of the divided retinal quadrants is taken on nitrocellulose membrane, treated with melanopsin antibody and incubated for 3 d at 4° C to increase the binding. The melanopsin antibody is a rabbit polyclonal (UF006), which recognizes a

sequence representing the 15 most N-terminal amino acids of the mouse melanopsin extracellular domain (Ecker et al. 2010, Hattar et al. 2002). The specificity of the melanopsin antibody was further confirmed by pre-adsorption by a blocking peptide.

The retinal whole mounts were incubated for 1 h in a cocktail solution to block non-specific staining. The cocktail solution included 10% normal donkey serum, 3% BSA and 0.3% Triton X-100 in PBS (pH 7.4), The whole mounts were incubated in a mixture of primary antibodies against melanopsin (diluted 1:7500) for 3 d. A negative control retina without Mel-SAP conjugate treatment was also processed in parallel. After 3 d of incubation, the retinas were washed in PBS for 5 × 5 min and the samples were again incubated in the appropriate secondary antibody (donkey anti-rabbit conjugated with Alexa Fluor-488 (1:500) for melanopsin for 1 h. The samples were washed again in PBS for 5 × 5 min and mounted using Vectashield containing 4', 6-diamidino-2-phenylindole (DAPI). DAPI binds to double-stranded DNA and labels the nuclei of all cells with blue fluorescence.

Morphometric measurements and analysis

The images of immunostained retinal whole mounts were captured on an epifluorescent microscope (AxioImager.Z1, Carl Zeiss, Gottingen, Germany) using a CCD camera (AxioCam MRm). The AxioImager microscope was equipped with ApoTome grid projection system which enabled us to capture images with increased contrast and enhanced optical resolution in z-axis. Briefly, the grid was projected on to the image by the reflected light. The image of the grid was shifted in 3 defined phases using a scanner in ApoTome. This reduced the background in the image and produced a stronger signal. Later, these grid images were offset against one another to get an optical section without the grid (manufacturer's specifications).

For the whole mounts that were used for measurement of dendritic stratification, 1 µm thickness of 15–20 optical sections in the z-axis were captured to include all labelled cells in the GCL. Contiguous frames were imaged to cover the entire retina, using arbitrary landmarks in each frame, such that all labeled cells were imaged and none of them was imaged more than once. The cells labeled for DAPI and melanopsin were counted in all the frames using ImageJ. The images were analyzed in ImageJ software (National Institutes of Health, USA) to differentiate between individual subtypes of mRGCs based on their dendritic stratification. The number of melanopsin-positive cells based on their stratification in different layers of retina were counted separately and data taken on Microsoft excel sheet for analysis. Care was taken not to consider scleral cells, blood arteries and veins.

OBSERVATIONS

In our pilot experiments, there was no evident loss of mRGCs on treatment with 100–400 ng/eye of Mel-SAP conjugate. However, an increase in Mel-SAP conjugate concentration from 600 ng/eye to 800 ng/eye has shown drastic reduction in mRGC total population. On treatment with 600 ng/eye of Mel-SAP conjugate, there was a reduction in average cell density to 52 cells/mm^2 as compared to 106 cells/mm^2, as found in control indicating 44.9% melanopsin cell ablation. Increase in immunotoxin Mel-SAP concentration to 800 ng/eye has yielded 8.5 cells/mm^2 resulting in 91% loss of melonpsin cells in a retinal quadrant (Table 1). The cell density values slightly vary as intravitreol injection is little tricky to conduct. Nevertheless, if average values of 2

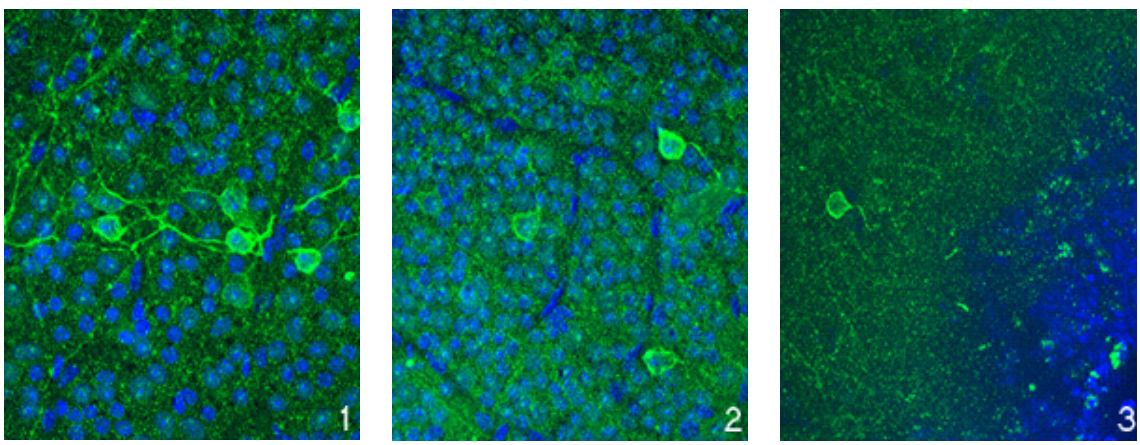
quadrants considered would also indicate the measurable ablation of mRGC and its subtypes (Figs 1–3).

M1 cells stratifying in OFF sublayer of inner plexiform layer showed an average of 18.18 cells/mm^2 upon 600 ng/eye of Mel-SAP treatment and 3.20 cells/mm^2 upon 800 ng/eye treatment of Mel-SAP conjugate compared to total cell count of control. M2 cells stratifying only in ON sublayer showed 26.67 and 3.53 cells/mm^2 upon 600 and 800 ng/eye treatment of Mel-SAP conjugate (Table 1). By assessing the cell ablation in ganglionic cell layer cells that were labelled with DAPI for the current investigation, we were able to confirm the specificity of the Mel-SAP conjugate. With an increase in Mel-SAP conjugate concentration from 600 to 800 ng/eye, the results showed a 43.3–65% loss (Figs 1–3). DAPI-stained ganglion cells were present in the control retina at a density of about 6000

TABLE 1: The cell density values of Mel-SAP conjugate treated and untreated (control) values in mice.

Mel-SAP conjugate concentration	Total	M1	M2 (non-M1cells)	DAPI cells
Control	106			6000.0
600 ng/eye quadrant 1	44.7	12.45	28.07	3683.7
600 ng/eye quadrant 2	59.3	23.92	25.27	3098.1
Average (% of ablation*)	52 (44.9%)	18.18 (80.7%)	26.67 (71.7%)	3400 (43.33%)
800 ng/eye quadrant 1	11.7	4.37	3.91	2024.7
800 ng/eye quadrant 2	5.12	2.04	3.16	2181.9
Average (% of ablation*)	8.5 (91 %)	3.20 (96.6%)	3.53 (96.25%)	2100 (65%)

* The percentage of mRGC ablation is calculated using the formula 100% of average cells.



Figs 1-3: Retinal whole mounts of melanopsin cells of mice stained with Alexa Fluor-488 conjugated to a secondary antibody and DAPI-stained nuclei as an apparent background. 1. Control untreated with Mel-SAP conjugate. 2. Whole mount of retinal quadrant treated with 600 ng/eye Mel-SAP conjugate showing cell ablation. 3. Whole mount of retinal quadrant treated with 800 ng/eye showing high ablation of cell loss.

cells/mm^2, whereas Mel-SAP conjugate-treated whole mount revealed an average of 3400 to 2100 cells/mm^2 with increasing immunotoxin concentrations of 600 to 800 ng/eye (Table 1).

DISCUSSION

In 1998, the melanopsin molecule was first discovered on frog skin, which was later shown to be localized to a small subset of retinal ganglion cells (RGCs) called melanopsin retinal ganglionic cells (mRGCs) (Provencio et al. 1998, 2000). These mRGCs are of 5 subtypes, ranging from M1 to M5, each with distinct and slightly overlapping morphological features (Reifler et al. 2015). Our study aims to selectively ablate these mRGC subtypes, specifically M1 and nonM1 cells, so that we can study the activity of 2 different melanopsin subtypes. Our observations showed no significant loss of mRGCs on treatment till 400 ng/eye. However, there was a sudden rise in ablation of melanopsin cells with increasing concentration of 600 ng/eye to 800 ng/eye Mel-SAP conjugate treatment. We further looked into the effect of immunotoxin on individual subtype loss of melanopsin cells. Although, we found the overall reduction of cell count in melanopsin cell population, there was no evident full loss of individual subtype of melanopsin cell. Instead, we observed a proportionate loss of melanopsin cell with increasing concentration of Mel-SAP conjugate application (Göz et al. 2008, Ingham et al. 2009).

M1 cells stratifying in OFF sublayer of inner plexiform layer showed 80.7% and 96.6% cell loss upon 600 and 800 ng/eye treatment of Mel-SAP conjugate compared to total cell count of control while M2 cells stratifying only in ON sublayer showed 71.7% and 96.25% ablation upon 600 and 800 ng/eye treatment of Mel-SAP conjugate. The results suggest that the percentage of cell ablation is dependent on concentration of Mel-SAP treatment and the cell ablation is random when compared between 2 individual subtypes of melanopsin cells. There is equivalent chance for all subtypes to get affected by the Mel-SAP conjugate as its primary antibody is polyclonal and binds selectively to C-terminal of melanopsin cells, which might be a conserved sequence present in all subtypes.

In the current study, we also verified the specificity of Mel-SAP conjugate by measuring the cell ablation in DAPI-stained cells of ganglionic cell layer. The loss of 43.3–65% mRGCs with increasing Mel-SAP conjugate concentration from 600 to 800 ng/eye clearly indicates the drug action being nonspecific. The primary melanopsin antibody is a rabbit polyclonal, so the drug targeting is compromised. However, the results can also be erroneous due to distortion in Mel-SAP conjugate link and deviate from what others have claimed (Ingham et al. 2009, Rollag et al. 2003). The Mel-SAP conjugate is extensively used till today to study behavioural deficits of non-visual response processes (Delwig et al. 2018, Lucas et al. 2020, Yamakawa et al. 2019). The present study suggests that the Mel-SAP conjugate is not specific to mRGC alone but affects several other types of ganglion cells, making it a non-specific drug.

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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF *MARSDENIA SYLVESTRIS* LEAF EXTRACT

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SUMMARY Antibacterial activity of different solvent-based leaf extracts of *Marsdenia sylvestris*, a medicinal plant commonly used in traditional medicine in India has been studied. The plant extract was tested against 5 bacterial pathogens, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus*. The results showed that ethanol and chloroform extracts were most effective against all tested pathogens and suggest that *M. sylvestris* can be used as a potential source of broad spectrum antibacterial agents for ayurvedic and pharmacological preparations.

Keywords: *Marsdenia sylvestris,* antibacterial activity, leaf extracts, agar well diffusion, DMSO.

INTRODUCTION

Marsdenia sylvestris (Retz.) P. I. Frost. (syn: *Gymnema sylvestre* (Retz.) R.Br. ex Sm.) (Apocynaceae) is a rain forest vine found in the tropical forests of central and southern India and also in Banda, Konkan, Western Ghats, Deccan and extending to parts of western and northern India (Saneja et al. 2010). It has long been known for the treatment and management of type 2 diabetes and is commonly named Gurmar, meaning 'sugar destroyer'. Its use has been documented in Ayurvedic medical texts for over 2000 y in the treatment of 'sweet' urine. In the United States, *Marsdenia* is gaining popularity for the treatment of type 2 diabetes among physicians familiar with alternative therapies (Leach 2007, Thakur et al. 2012).

According to the Charaka Samhita, this plant has the ability to remove bad odours from breast milk and has an appetizing effect. It is also beneficial as a purgative and for treating eye ailments. The extract from the leaves and flowers is useful for the eyes, while the bark is given for diseases caused by vitiated kapha (Saneja et al. 2010). In Ayurveda, this plant has been described as having several properties such as acrid, alexipharmic, anodyne, anthelmintic, antipyretic, astringent, bitter, cardiotonic, digestive, diuretic, emetic, expectorant, laxative, stimulant, stomachic and uterine tonic (Diwan et al. 1995). It is useful in treating various conditions including amenorrhea, asthma, bronchitis, cardiopathy, conjunctivitis, constipation, cough, dyspepsia, haemorrhoids, hepatosplenomegaly, inflammations, intermittent fever, jaundice, and leukoderma (Thakur et al. 2012). The root of the plant can be used as an emetic to remove phlegm, and it can be applied externally to treat insect bites. However, the species-specific antibacterial activity of *M. sylvestris* is still obscure. Our study focuses on different solvent extracts of *M. sylvestris* to various types of bacterial mono-

cultures viz., *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus* to assess its effects.

MATERIAL AND METHODS

Materials were collected from the Siddarabetta (13.5651° N, 77.1420° E) of Tumkur district, and maintained in the garden of Maharani Cluster University, Bengaluru. The plant samples were authenticated at Regional Ayurveda Research Institute, Bengaluru.

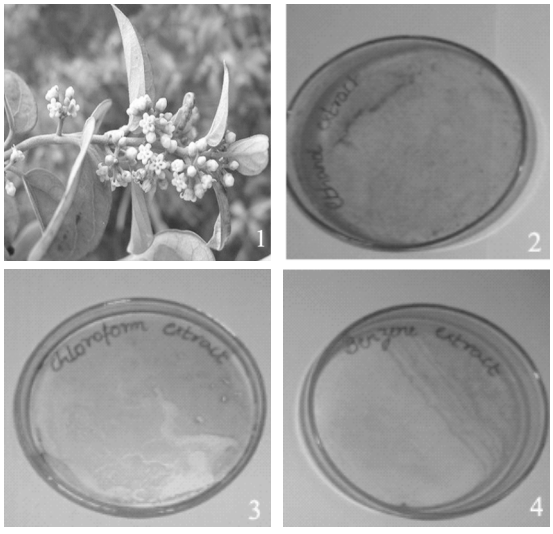
Five human pathogenic bacteria were selected to evaluate the antibacterial activity. Gram-negative bacteria such as *E. coli, K. pneumoniae* and *P. aeruginosa,* as well as Gram-positive bacteria, *B. subtilis* and *S. aureus*. The colonies showing standard characters were partially picked up and subjected to various microscopic characters like Gram's staining, motility, capsule and spore formation as per the standard laboratory procedures (Collins & Lyne 1970). All 5 bacterial pathogens were selected and confirmed based on their colony organization, microscopic features along with suitable biochemical tests. The bacterial strains used were pure isolates obtained from the Department of Microbiology, Maharani Cluster University and were maintained in nutrient agar.

The leaves of *M. sylvestris* were thoroughly washed under running tap water, dried using paper towels, and shade-dried again. The dried leaves were then crushed to a fine powder using a mixer grinder. The dried leaf powder was subjected to soxhlet extraction using benzene, chloroform, and ethanol in sequence (Sinha et al. 2010). The resulted liquid extracts were collected, filtered and evaporated using a rotary evaporator to obtain a semisolid or dried crude leaf extract. The latter was resuspended in an inert solvent, DMSO, and dissolved to reach a final concentration of 100 mg/ml. The extracts were stored at 4° C until further use.

To determine the antimicrobial activity of extracts, the agar well diffusion technique was used (Manni & Sinsheimer 1965). Nutrient agar plates were first seeded with 0.1ml of an overnight culture of each bacterial strain. A 24 h broth culture of each bacterium was then used to seed sterile molten agar, which was allowed to solidify at room temperature. Wells were then made in the agar using a sterile standard cork borer, and a 100 mg/ml solution of extract was added to each well. The bacterial plates were then incubated at 37° C for 24 h, after which the diameter of the zones of inhibition was measured in mm using Hi Antibiotic Zone Scale-C. A standard antibiotic strip of ciprofloxacin (5 µg/disc) for each bacterium along with DMSO was used as a negative control. The minimum inhibitory concentration (MIC) was determined using the broth dilution method. Initially, 0.6 g of each extract was dissolved in 300 ml sterile nutrient broth, resulting in an initial concentration of 2000 µg/ml. This was then subjected to two-fold serial dilution to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.5, and 15.6 µg/ml. Different concentrations of leaf extract in benzene, chloroform, and ethanol were tested separately for each bacterium, and the inhibition zone of microbial growth in the plates containing test solutions was judged by comparison with blank control plates.

OBSERVATIONS

The plant extracts were prepared using solvents such as benzene, chloroform and ethanol (Figs 1–4). The antibacterial activity was evaluated against 5 bacterial pathogens, *E. coli, K. pneumoniae, P. aeruginosa*, *B. subtilis,* and *S.*



Figs 1–4: Antibacterial activity of *M. sylvestris*. 1. A flowering twig. 2. Ethanol extract. 3. Chloroform extract. 4. Benzene extract.

aureus using the agar well diffusion method. The results showed that the ethanol extracts of *M. sylvestris* exhibited the highest antibacterial activity against all 5 bacterial strains as compared to benzene extracts. Both Gram positive bacteria, *S. aureus* and *B. subtilis* showed maximum sensitivity of 25 mm and 24 mm of zone of inhibition (ZOI) to a minimum inhibition concentration of 15.6 µg/ml of *M. sylvestris* leaf extract (Table 1). While Gram negative bacteria like *P. aeruginosa* exhibited 22 mm of ZOI, *E. coli* and *K. pneumoniae* showed closer to 20 mm of ZOI. The zone of inhibition was comparable to that of the standard antibiotic ciproflaxin (Ci).

The chloroform solvent-based leaf extracts of *M. sylvestris* showed a similar trend as that of ethanol extract. However, MIC required is quite higher than ethanol extract to inhibit the growth of bacteria. There was 23 mm zone of inhibition in *S. aureus,* 22 mm in *P. aeruginosa* and 21 mm in *B. subtilis.* The benzene solvent-based leaf extract displayed a maximum of 18 mm ZOI against *B. subtilis* and against *S. aureus* and *P. aeruginosa* exhibited 14 mm ZOI each indicating no barrier in Gram staining based differentiation of bacteria (Table 1). These findings suggest that leaf extract of *M. sylvestris* possesses broad spectrum antibacterial activity.

DISCUSSION

Numerous studies have been carried out to investigate the ability of various substances, particularly of plant origin, to act against microbes. Plants are a valuable source of potential therapeutic agents, as they contain active compounds known as secondary metabolites that have been linked to antibacterial properties. For instance, tannin-rich plants have antibacterial potential due to their ability to react with proteins and create stable, water-soluble compounds, which damage the cell membranes of bacteria, leading to their demise (Segarajah et al. 2011).

TABLE 1: Antibacterial activity of different solvent-based leaf extracts of *M. sylvestris* against 5 pathogens.

Microorganism	Benzene extract		Chloroform extract		Ethanol extract		Cip	DMSO
	ZOI	MIC	ZOI	MIC	ZOI	MIC		
E. coli	-	-	14	500	20	125	20	-
K. pneumoniae	-	-	16	500	19	250	20	-
P. aeruginosa	14	>1000	22	125	22	62.5	20	-
B. subtilis	18	500	21	250	24	15.6	22	-
S. aureus	14	1000	23	62.5	25	15.6	23	-

Phenolic compounds, such as flavonoids, are also well known for their antiviral, antimicrobial, and spasmolytic properties (Vani et al. 2016). Moreover, alkaloids isolated from plants are found to have antimicrobial qualities. The present study reveals that *M. sylvestris* has antimicrobial activity, and its leaf extracts contain phytochemical compounds, such as alkaloids, saponins, tannins, flavonoids, and glycosides, that may act alone or together to inhibit bacterial growth and contribute to their strong antibacterial activity (Shivanna & Raveesha 2009).

Overall, plant-derived antibiotics tend to be more effective against Gram-positive bacteria than Gram-negative ones, due to the lipopolysaccharide layer present on the outer surface of the latter, which hinders access to the peptidoglycan layer of the cell wall (Ye et al. 2000). The current study found that the crude extracts from the leaves of *M. sylvestris* were more effective against Gram-positive bacteria than Gram-negative ones indicating their potential use in controlling infectious diseases caused by both types of pathogens. The effectiveness of the crude extracts can vary depending on the solvent, ambient temperature, bacterial strains, season variations and extraction methods used (Khan et al. 2019). For example, some have reported that *M. sylvestris* extracts are effective against *B. subtilis* and *S. aureus*, but not against *E. coli*, while others suggest that chloroform and ethanol extracts may be useful in treating infectious diseases (Porchezhian & Dobriyal 2003). In the present study, ethanol extracts were found to be most effective against *S. aureus, P. aeruginosa,* and *B. subtilis*, but also exhibited inhibition zones against *E. coli*. The ciprofloxacin-treated ones showed 20 mm ZOI which is equivalent to ethanol based leaf extract of *M. sylvestris* treated against *E. coli*. Furthermore, these findings suggest that *M. sylvestris* has potential as a broad spectrum drug candidate for preventing and treating infections caused by *E. coli* and *S. aureus*. The broad spectrum antibacterial activity of *M. sylvestris* could be attributed to the presence of various phytochemicals like saponins, tannins, flavonoids, and alkaloids (Khan et al. 2019, Sukesh et al. 2011). These compounds have been reported to have antibacterial properties and could be responsible for the observed antibacterial activity of *M. sylvestris* extract. The findings of this study suggest that ethanol and chloroform based leaf extracts of *M. sylvestris* could be used as a potential source of antibacterial and antimicrobial agents for the development of new drugs (Srinivasan & Kumaravel 2016). However, further studies are required to identify the active compounds responsible for the observed activities and to determine the mechanism of action.

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RESEARCH ARTICLE

KARYOTYPE BASED SYSTEMATIC CHARACTERIZATION OF SMALL SUPERNUMERARY MARKER CHROMOSOME IN HUMAN CLINICAL SAMPLES: A CASE STUDY

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SUMMARY Small supernumerary marker chromosomes (sSMCs) are extra abnormal chromosomes found in the genome. Although sSMCs account for less than 0.043% of newborns, yet is the most prevalent anomaly associated with inborn mental retardation. In the present study, 100 couples with recurrent miscarriages were investigated for chromosomal abnormalities. Out of 200 subjects, 34 cases of abnormal karyotypes were identified and categorized. Among them, 30 cases were showing chromosomal aberrations, while 4 cases had numerical changes. In the latter, 2 male cases of 27–28 y show the presence of sSMCs. Both cases were found to be hereditary. A comparative investigation indicated that these sSMCs could develop via paracentric inversion, fragmentation, or non-disjunction. It was noticed that in cases with sSMC, 50% of the gametes produced were normal, indicating that gamete sorting preceding IVF (GIFT and ZIFT) could possibly lead to healthier offspring. Further investigation of sSMCs via microarray, FISH or NGS could help in both the systematic characterization of sSMcs and the illustration of novel mechanisms of chromosomal abnormalities.

Keywords: Human karyotype, small supernumerary marker chromosome, paracentric inversion, fragmentation, non-disjunction, FISH, NGS.

INTRODUCTION

Since Griffith (1928) identified DNA as a genetic material, and Hershey & Chase (1952) confirmed this discovery, scientists have started to look into how DNA is passed down from parents to children. Although chromosomes were first discovered by Walther Flemming in 1882 (Paweletz 2001), it was not until the revolutionary work of Morgan that the chromosomal theory of inheritance was confirmed (Bridges 1916, Benson 2001, Klug et al. 2019). These findings have led to the establishment of cytogenetics as a prominent field of cell biology. The development of electron microscopy by Knoll & Ruska (1932) and Ruska (1987) has further strengthened our understanding of cytogenetics. In 1960, Peter Nowell and David Hungerford discovered Philadelphia chromosomes in the karyotype of chronic myeloid leukaemia using cytogenetic methods (Nowell & Hungerford 1960). Later, in

1973, Janet Rowley employed quinacrine fluorescence and G-banding on such patients to illustrate that Philadelphia chromosomes are a result of crossing over or translocation between chromosomes 9 and 22 (Rowley 1973). These studies have further inculcated the interest of researchers in the field of cytogenetics.

Chromosomal abnormalities or genetic anomalies may occur in several newborns, including both genomatic mutations and chromosomal aberrations (Zarocostas 2006, Soler et al. 2017, Bharadwaj et al. 2021). Among all chromosomal abnormalities, the least characterised are, small supernumerary marker chromosomes (sSMCs) (Liehr et al. 2012). These are extra, abnormal chromosomes that constitute copies or parts of one or more chromosomes (Baldwin et al. 2008, Liehr et al. 2012). sSMCs are unique abnormalities as their origin cannot be investigated by conventional banding techniques (Liehr et al. 2008, Saberzadeh et al. 2019). It is estimated that 0.043% of livebirths and 0.072% of prenatal cases involve an extra structurally deficient chromosome of unknown origin. They occur 7 times more frequently in patients with mental impairments (Liehr & Weise 2007). Clinical diagnostics involve karyotyping, which is considered a "gold standard" method in human cytogenetic investigations of the genome (Polopalli et al. 2016). The development of the multicolour FISH (M-FISH) assay and its combination with genomic karyotype has significantly improved the identification of sSMCs (Liehr et al. 2013). The current study aims to investigate the presence of sSMCs and explore their mechanism of development in clinical samples from 100 couples with a history of miscarriages.

MATERIALS AND METHODS

Hundred couples with a history of recurrent spontaneous miscarriages were investigated over a period of 3 y during 2019–2022 after obtaining permission from the University Ethical Committee and consent from patients. Peripheral whole blood (5 ml) was collected from all the subjects (both male and female of 100 couples). Lymphocyte culture method was performed for each sample collected and investigated via karyotyping to illustrate their chromosomal organization.

Karyotyping of isolated peripheral blood lymphocytes

Freshly obtained isolated lymphocyte samples were processed within 24 h under strict aseptic laboratory conditions. The methodology employed in the study was based on the improvisation of the technique outlined in the "AGT Cytogenetics Laboratory Technical Manual" (Barch et al. 1997), which is a widely recognised manual for conducting cytogenetic research, developed by the Association of Genetics Technologists (AGT) (Boyum 1968, 1976, Fotino et al. 1971, Boyum et al. 1991, Barch et al. 1997).

***Cell culturing using RPMI-1640*:** 0.5 ml of whole blood was added to 5 ml of RPMI-1640 complete media, containing 100 mg/ml 10 µl penicillin antibiotics, in 15 ml falcon tube. 200 mM of 100 µl L-glutamine was added, followed by addition of 100 µl of phytohemagglutin M. The culture was mixed and incubated at 37° C for 70 h. The falcon tubes were placed at an angle of 45°, for the culture to grow effectively.

***Pre-harvesting of samples*:** The culture tube was then poured with 100 µl of colcemid 0.1 µg/ml following 70 h of incubation. The combination was then incubated for a further 1 h at 37° C.

***Harvesting of samples*:** The culture tubes were centrifuged at 1000 rpm for 10 min at 25° – 27° C, until a clear pellet is produced. After discarding the supernatant, 5.0 ml of 0.56% potassium

chloride (hypotonic solution) was added to the pellet. The tube was incubated in pre-warmed water bath at 37° C for 25 min. A fixative solution was prepared by combining 3 parts of methanol with 1 part of glacial acetic acid, and subsequently added under chilled conditions. The tubes were centrifuged after incubation at 1000 rpm for 10 min. The supernatant was discarded and pellet resuspended in 7 ml of chilled fixative. The fixative centrifugation step is repeated until a clean white pellet begins to appear. Once a clear pellet is obtained, it is dissolved in 2 ml of fixative and stored at 4° C.

***Slide preparation and Giemsa staining*:** A grease free slide was taken and 2–3 drops of pre-fixed culture was dropped down from ≈1–2 feet above. Dropping of cells from the height of about 1–2 feet is important to get good quality metaphase spread. These slides are then dried on a slide warmer at 90º C and placed in dry oven for 1 h. The heat-fixed slide was stained with Giemsa for GTG banding. Following staining, slide was gently rinsed by double distilled water and dried. The image of Giemsa banded chromosome was captured via Leica fluorescence microscope, cytovision version 7.4 was used to capture the metaphase images. Photomicrograph of each chromosome at 2000 x to 3000 x magni-fications were cut out and arranged in descending order to form the karyotype.

Comparative karyotype analysis

The karyotype of 200 subjects (in triplet) for each member of the study was specifically monitored. A normal female karyotype was abbreviated as (46, XX) while for a normal male as (46, XY). An in-depth analysis of the karyotype obtained assisted with comparative analysis of characteristic chromosomal traits was specifically able to detect polyploidy, aneuploidy, translocations, inversions, ring chromosomes and sSMCs. Through comparative analysis of the karyotype obtained with regard to similar findings in other studies, attempts were made to illustrate the mechanism for the development of different sSMCs (Liehr et al. 2008, Liehr 2013, Shakoori 2017).

OBSERVATIONS

Karyotyping of isolated peripheral blood lymphocytes

Slides were prepared and stained by using Giemsa staining technique. There were 2 cases of sSMCs (Nos 69 and 117) with aberrant karyotypes that were clinically detected (Table 1, Fig. 2).

Comparative karyotype analysis

Each of the 200 participants in the study were carefully monitored in respect of their karyotypes (Fig. 1). The karyotype of a typical female was shortened as (46, XX), and that of a normal male as (46, XY). Potential development processes for sSMCs was shown in Figs 4–6.

DISCUSSION

Out of 200 samples (each in triplet) of 100 couples under investigation, 34 (17%) samples were found with abnormal karyotypes (Fig. 1). It indicated that the chromosomal abnormalities could be the possible reason for recurrent spontaneous miscarriages as advocated by Kim et al. (2010). In order to increase the sensitivity of the karyotype, it was preceded by lymphocyte culture method.

It was also noticed during the study that more than 60% cases of chromosomal abnormalities were reported in couples of over 30 y of age, thus such abnormal karyotypes could be the possible result of late marriages or ageing (Table 1). Among abnormal karyotypes, 88% (30) of cases were of chromosomal aberrations while 12% (4)

TABLE 1: Cytological analysis of clinically identified abnormal human karyotypes.

S.No.	Case No.	Age in years/Sex	Clinical symptom	Interpretation
1.	08	32/F	Recurrent pregnancy loss	46,XX,add (17)(p13.1)
2.	16	29/F	Repeated spontaneous abortion	46,XX, inv(9)(p11q12)
3.	18	27/F	Recurrent pregnancy loss	45,XX,t(13;14)(q10;q10)
4.	28	31/F	Recurrent pregnancy loss	46,XX,15ps+,22ps+
5.	38	29/F	Repeated spontaneous abortion	45,XX,rob(14;22) (q10;q10)
6.	56	38/F	History of abortions	46,XX,t(2;3)(p25;p21)
7.	62	26/F	History of abortions consanguineous marriage	46,XX,1qh+
8.	66	29/F	History of abortions	46,XX,inv(9)(p11q12)
9.	**69**	**27/F**	**History of abortions**	**47,XY,+mar**
10.	76	26/F	Recurrent pregnancy loss	45,XX, t(13;14)(q10;q10)
11.	80	31/F	Recurrent pregnancy loss	47,XXX
12.	84	32/F	History of abortions	46,XX,22ps+
13.	86	31/F	History of abortions	45,XX,rob(14;21)(q10;q10)
14.	91	28/M	History of abortions	46,XY,14ps+
15.	93	37/M	History of abortions	46,XY,inv(9)(p11q12)
16.	102	33/F	Repeated spontaneous abortion	46,XX,14ps+
17.	103	35/M	Recurrent pregnancy loss	46,XY,t(2;22)(p21;p13)
18.	106	24/F	Repeated spontaneous abortion	46,XX,inv(9)(p11q12)
19.	110	30/F	Recurrent abortions	46,XX,22ps+
20.	115	32/M	History of abortions	46,XY,inv(Y)(11.2q11.23)
21.	**117**	**28/M**	**History of abortions**	**47,XY,+mar**
22.	120	26/F	History of abortions	46,XX,22ps+
23.	123	32/M	Repeated spontaneous abortion	46,XY,15ps+
24.	130	35/F	Recurrent pregnancy, Advance maternal age	46,XX,t(3;10) (p10;p10)
25.	137	33/M	Recurrent pregnancy loss	46,XY,inv(Y)(p11.2q11.23)
26.	149	40/M	Repeated spontaneous abortion	46,XY,t(7;10)(p21.3;q21.1)
27.	157	40/M	History of abortions	46,XY,15ps+
28.	161	32/M	Recurrent pregnancy loss	46,XY,t(4;5)(q22;p15.3)
29.	168	28/F	Repeated spontaneous abortion	46,XX,t(4;13)(p15.2;q22)
30.	174	25/F	Recurrent pregnancy loss	46,XX,t(4;18)(p15.2;p11.3)
31.	180	39/F	Spontaneous abortion, advance maternal age	46,XX,t(10;11) q11.2;q25)
32.	187	32/M	Repeated spontaneous abortion	45, XY,t(13;14)(q10;q10)
33.	191	31/M	Recurrent pregnancy loss	46,XY,inv(Y)(p11.2q11.23)
34.	199	45/M	Repeated spontaneous abortion and advance maternal age	46,XY,t(5;12)(q22; q13)

p, Smallest arm; q, Longest arm; add, Addition; inv, Inversion; t, Translocation; s+, Satellite; h+, Heterochromatin; +mar, Marker; rob, Robertsonian.

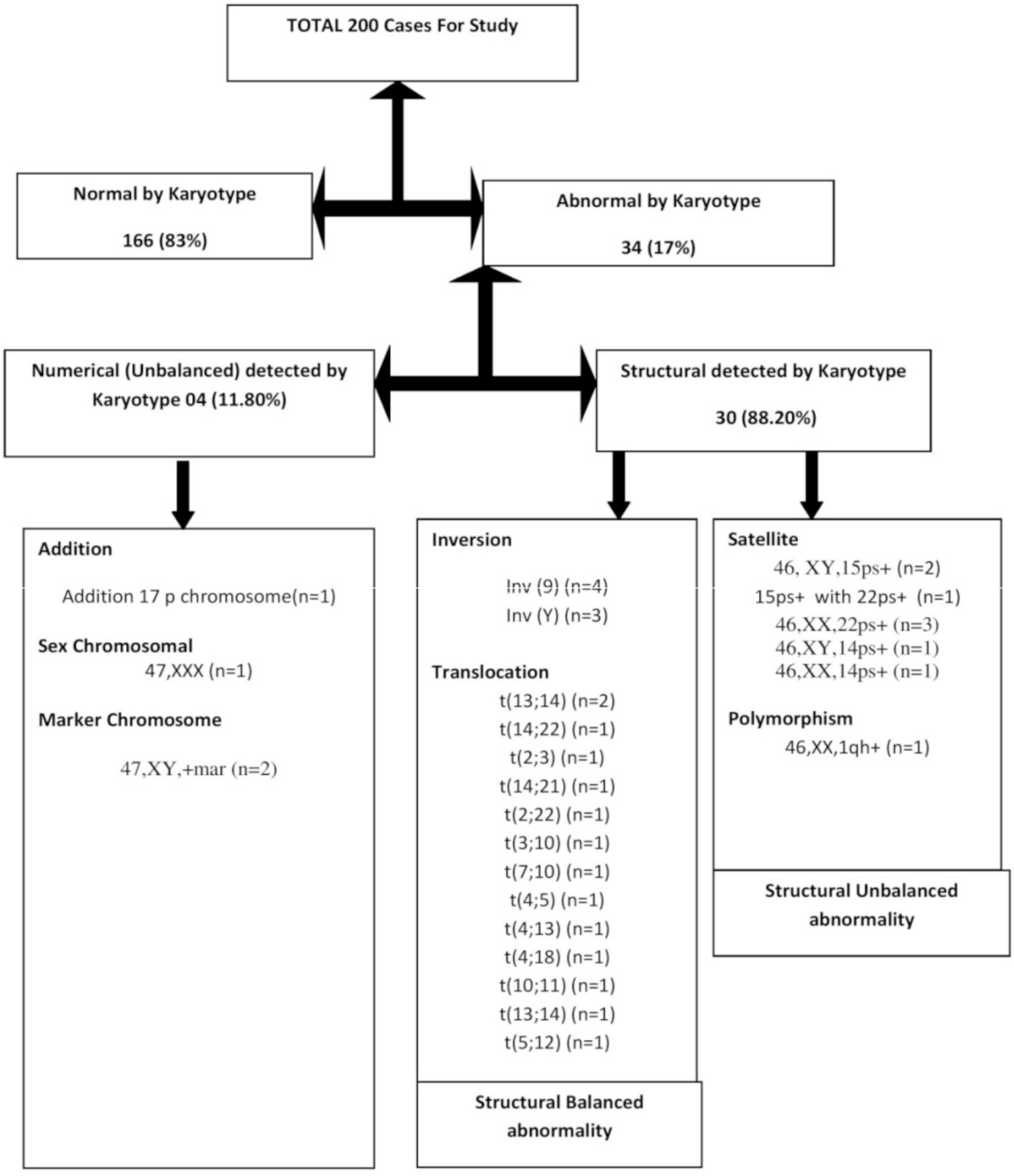

Fig.1: Frequency of different chromosomal abnormalities as observed in collected clinical samples. (n stands for number of cases).

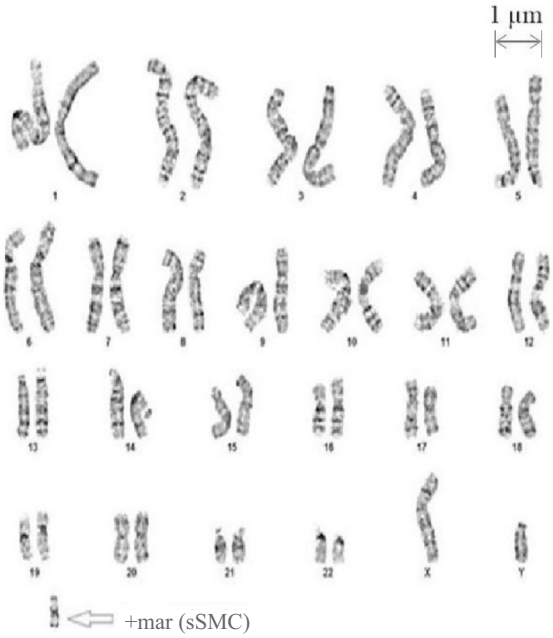

Fig. 2: The small supernumerary marker chromosome (+mar, sSMC) in clinical sample coded as case 69, through karyotype analysis.

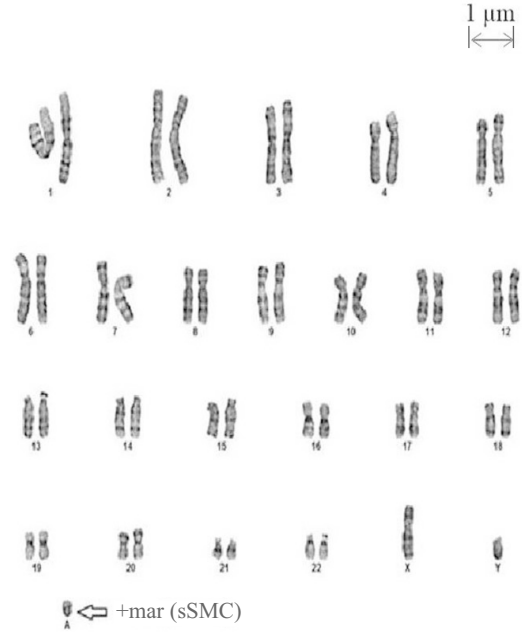

Fig. 3: The small supernumerary marker chromosomes (+mar, sSMC) in second clinical sample coded as case 117, through karyotype analysis.

of cases had numerical changes (Fig. 1). The chromosomal aberrations were predominantly constituted by translocation 41% (14) of cases, satellite DNA 26% (9) of cases, inversions 20% (7) of cases. Among cases of numerical changes, only in 6% (2) of cases sSMCs were observed (Fig. 2). Occurrence of sSMCs could be considered as one of the rarest chromosomal abnormalities as postulated by Karamysheva et al. (2020) and Liehr et al. (2012).

Two cases of sSMCs were studied in-depth, and the results were compared with typical karyotypes. According to Liehr et al. (2008), paracentric inversion occurs when a chromosomal tract is inverted away from the centromere, rotated through 180°, and then the pieces are reassembled. Paracentric inversions result in the development of a dicentric bridge and an acentric fragment if crossing over occurs within the inversion loop during meiosis (Figs 4–6). The centromere is missing from this acentric sSMC segment. Baldwin et al. (2008) noted that radiations (X-rays, γ-rays, β-rays, etc.) might produce fragmentation due to their mutagenic or clastogenic properties. Meiotic non-disjunction of sister chromatids may produce (n/C+1) gametes, which, if fertilized by a normal gamete, may produce an offspring with a karyotype that includes sSMC. Most researchers agree that non-disjunction is the root cause of the problem when it comes to sSMCs (Kurtas et al. 2019).

Due to the presence of sSMCs critical housekeeping genes could be lost during meiosis I. This could lead to spontaneous abortions of the developing foetus. It is to be further noticed in case of either fragmentation or paracentric inversions leading to sSMCs formation, 50% of

Paracentric inversion leading to sSMCs.

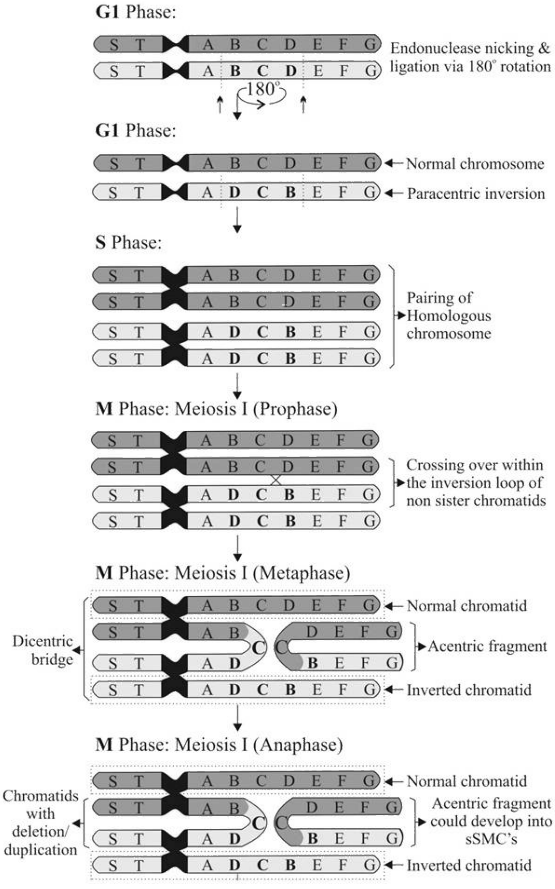

Fig. 4: Possible mechanisms of development of sSMC. a) Paracentric inversion within a chromosomal arm in a homologous pair, leading to a possible acentric sSMC.

Fragmentation of chromosome leading to sSMCs.

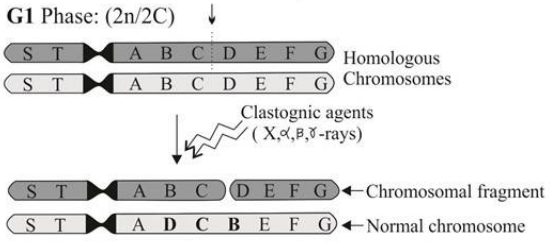

Fig. 5: Possible mechanisms of development of sSMC. Fragmentation of chromosomal arm via exposure to clastogenic agents (X-rays, α-rays, β-rays, γ-rays, etc), leading to a possible sSMC.

Non-disjunction of sister chromatids leading to sSMCs.

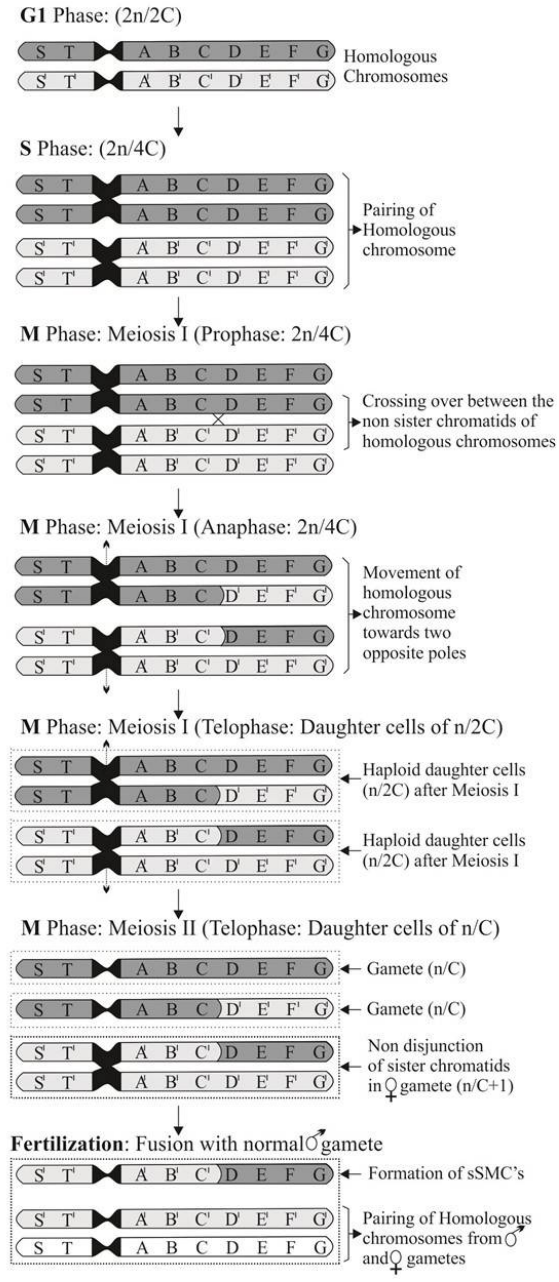

Fig. 6: Possible mechanisms of development of sSMC. Non-disjunction of sister chromatids could lead to (n/C+1) gametes which could fertilize with a normal gamete leading to a possible sSMC development within the offspring karyotype.

the gametes produced are of normal karyotype. This indicated that gamete sorting for normal karyotype followed by artificial insemination (GIFT) or in vitro fertilization could act as a method of choice in such patients to have a healthier offsprings.

The findings of the present study suggest that cytogenetic approaches like karyotype analysis might be helpful in identifying prenatal anomalies in developing foetuses by amnio-centesis due to the fact that it has been demonstrated to be accurate to a greater extent than 99.9% (Bridge 2008). We feel that the array comparative genomic hybridization (aCGH), fluorescent in situ hybridization (FISH), analysis of GTG-labeled metaphase chromosomes and its scrutinization using microarray CGH, along with next generation sequencing (NGS), are all methods that could be used to further characterize sSMCs (Liehr et al. 2008, Liehr 2013, Shakoori 2017). This might offer insight on the likely evolutionary trend of the human genome, the genesis of chromosomes, the diversifications that have occurred in them, as well as to explain the advent of B-chromosomes or sSMCs. Despite the fact that karyotypes offer a visual genome-wide screen for chromosomal abnormalities, their resolution is only between 5 and 10 megabases, which means they are unable to identify minor chromosomal or point mutations, offers an illustration of this (Bridge 2008).

sSMCs has the potential to forecast the aetiology of these prenatal anomalies, their pattern of inheritance, and the causes for recurring abortions. If a parent (male or female) is found to have an aberrant karyotype, then it is possible that in vitro fertilisation (IVF) will be suggested to that parent in order to have children with healthier karyotypes. During IVF, cytogenetic analysis may serve as a useful technique for monitoring the progression of any chromosomal abnormalities in the developing foetus. sSMCs, which are uncommon and poorly characterized, may shed light on hitherto unknown processes underlying chromosomal aberrations. It would make genetic counseling much easier to get and would make it possible for parents to pass on better genes to their children.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DECLARATION

The study has been conducted at the Department of Biochemistry, in collaboration of Department of Obstetrics & Gynecology of Rama University, Kanpur, U.P., India on the basis of clinical history of the patients' family and through clinical reports provided. A prior approval was taken from the University Ethical Committee, and a written consent, in the vernacular language, was also obtained from all the participants.

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