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RESEARCH ARTICLE

KARYOTYPIC STUDIES ON ROSE APHIDS FROM THE MANDI REGION OF HIMACHAL PRADESH

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SUMMARY The karyotypes of aphids, *Macrosiphum euphorbiae*, *Rhodobium porosum* and *Sitobion rosaeiformis* infesting rose plants from Mandi, Himachal Pradesh have been studied. The diploid chromosome number in these species ranged from 10 to 18. The chromosomes are holocentric. The metaphase plates were used for the measurement of the chromosomes and the total complement length of each species were calculated. The relative lengths of the chromosomes of each species were used for idiogram preparation.

Keywords: Aphids, karyotypes, holocentric chromosomes.

INTRODUCTION

Aphids (Homoptera: Aphididae) are small, soft bodied insect pests of cultured and uncultured crops and are of various colours and ranging in size from 0.7 to 1.5 mm (Dixon 1998). They damage the plants by sucking their phloem saps with their stylets and also act as vectors for transmitting viruses (Will & Vilcinskas 2015). Being one among the most important insect pest groups, they show polyphenism, host alternating behaviour, and are polyphagous.

251 species of aphids have been described taxonomically from Himachal Pradesh by Ghosh (1986). Chromosomes of aphids infesting roses belonging to the genera, *Chaetosiphon, Macrosiphon, Metopolophium, Myzaphis* and *Sitobion* from Himachal Pradesh were previously studied by Kumari & Gautam (2014, 2019).

Rose is an ornamental plant that has extraordinarily beautiful and eye-catching flowers. Besides ornamental value, the petals of these flowers are a source of rose essential oil, the "liquid gold", a very valuable natural raw material, used especially in perfumes, cosmetics, aromatherapy and culinary practises (Feng et al. 2010). Rose absolute and essential oils contain high levels of phenolics and demonstrate strong antibacterial activity (Ulusoy et al. 2009). Chromosomal studies of rose aphids are essential to comprehending the biology, reproductive strategies and evolutionary adaptations which can result in more efficient pest management strategies and improved knowledge of plant-insect interactions.

The present paper deals with the karyotype analyses of 3 species of aphids namely, *Macrosiphum euphorbiae* (Thomas)*, Rhodobium porosum*

(Sanderson) and *Sitobion rosaeiformis* (Das) infesting *Rosa* spp. from Mandi region of Himachal Pradesh.

MATERIALS AND METHODS

The materials were collected from different places of the Mandi district of Himachal Pradesh which is located in the northwest Himalayas between 31°42' to 42.98" N and 76°55 to '57.83" E at an average altitude of 1,044 above sea level.

The rose plants were observed for aphid infestation and the aphids were collected from the young shoots, leaves and floral buds. The apterous adults were preserved in ethyl alcohol after collection and then were identified using the keys developed by Blackman & Eastop (1984). Somatic embryonic tissue from parthenogenetic females was used for chromosomal studies. These females were dissected by puncturing the posterior end of the abdomen for embryos. Only young embryos without any eye pigment were taken for chromosome preparation. Embryos were pretreated in 0.7% tri-sodium citrate solution for 25–30 min. This helps in clearing cytoplasm and softening of tissue. After pretreatment embryos were fixed in acetic-ethanol (1:3) solution for 15–20 min at room temperature. Then, squashing of the embryos was done by putting a drop of 45% acetic acid on pretreated embryos for 3–5 min. Both the slides and cover slips were stained in 2% Giemsa solution for 20–25 min and mounted in DPX.

The slides were observed with the binocular microscope to find the well spread plates of chromosomes and the photographs were taken. The actual lengths of chromosomes were measured using an ocular micrometer. The total complement length and the relative lengths of chromosomes were calculated from the actual lengths. The idiograms were prepared based on relative length data.

OBSERVATIONS

M. euphorbiae

Aphids of this species were light green and were collected from the apical shoots and ventral surfaces of the leaves of a rose plant. The diploid chromosome number in this species was found to be 10 (Fig.1). The chromosomes are holocentric. The mean length of chromosomes ranged from 0.68 µm ± 0.06 S.E. to 2.63 µm ± 0.14 S.E. (Fig. 2). The total complement length was 14.20 µm ± 0.69 S.E. The relative lengths of chromosomes ranged from 4.79 µm ± 0.39 S.E. to 18.52 µm ± 0.68 S.E. The idiogram shows a gradual decrease in the length of chromosomes (Fig. 3).

R. porosum

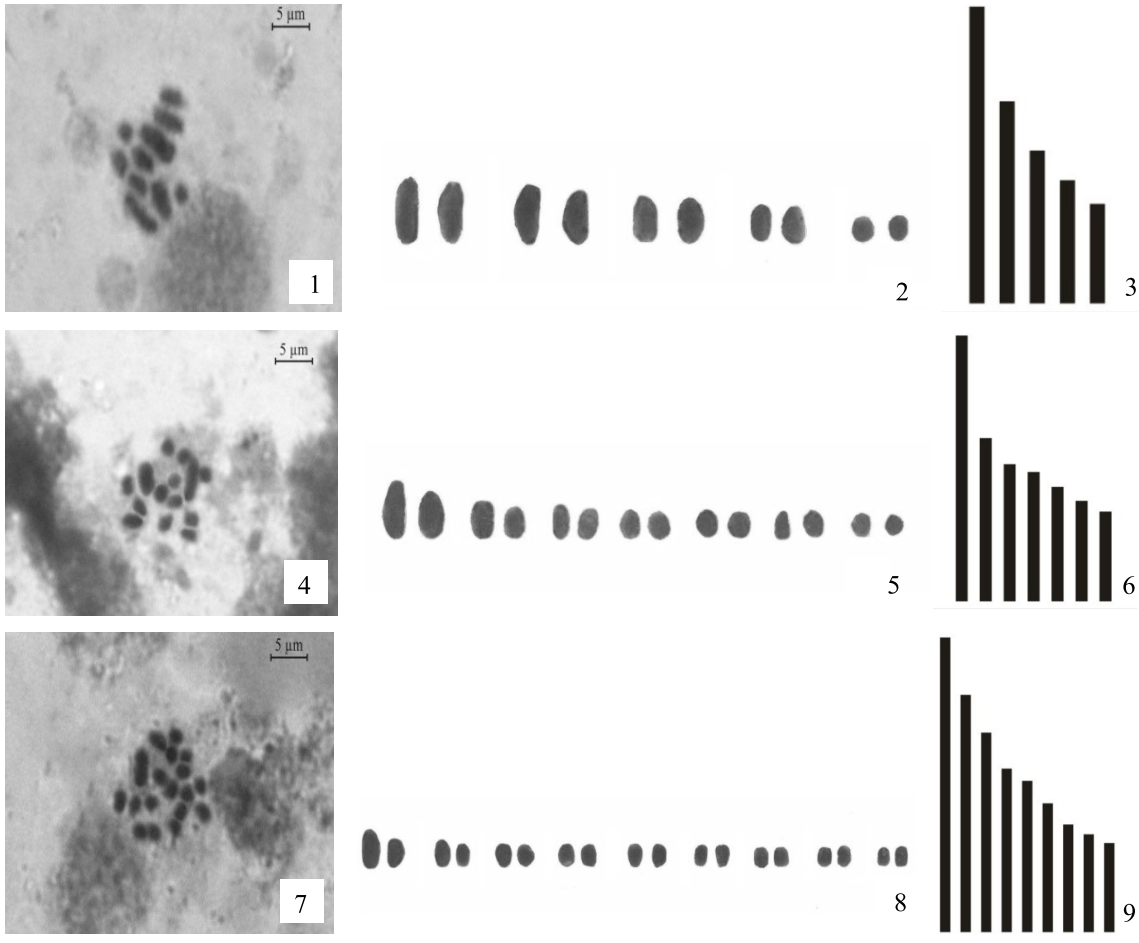
Aphids of this species were yellowish green to dark green and were collected from the apical shoots and floral buds of a rose plant. The diploid chromosome number in this species was found to be 14 (Fig. 4). The chromosomes are holocentric. The mean length of chromosomes ranged from 0.63 µm ± 0.03 S.E. to 2.03 µm ± 0.21 S.E. (Fig. 5). The total complement length was 14.12 µm ± 1.00 S.E. The relative lengths of chromosomes ranged from 4.46 µm ± 0.22 S.E. to 14.37 µm ± 1.45 S.E. The idiogram shows a gradual decrease in length of the chromosomes (Fig. 6).

S. rosaeiformis

Aphids of this species were light green to light orange and were collected from the floral buds of a rose plant. The diploid chromosome number in this species was found to be 18 (Fig.7). The chromosomes are holocentric. The mean length of chromosomes ranged from 0.57 µm ± 0.03 S.E. to 2.10 µm ± 0.12 S.E. (Fig. 8). The total complement length was 19.52 µm ± 0.93 S.E. The relative length of chromosomes ranged from 2.92 µm ± 0.12 S.E. to 10.76 µm ± 0.48 S.E. The idiogram shows a gradual decrease in the length of the chromosomes (Fig. 9).

DISCUSSION

In all 3 aphid species studied here the chromosomes are holocentric. They have 3 different chromosome numbers ranging from 2n = 10 to 18. The lowest diploid chromosome number recorded was 10



Figs 1–9: Karyotypes of aphids. 1–3. *M. euphorbiae*. 1. Somatic chromosomes. 2. Karyotype. 3. Idiogram. 4–6. *R. porosum* 4. Somatic chromosomes. 5. Karyotype. 6. Idiogram. 7–9. *S. rosaeiformis.* 7. Somatic chromosomes. 8. Karyotype. 9. Idiogram.

in *M. euphorbiae* and the highest was 18 in *S. rosaeiformis*. In *R. porosum* the diploid chromosome number is 14.

The diploid chromosome number of 10 reported here for *M. euphorbiae* is in conformity with the earlier reports by Devi & Gautam (2012), Gautam & Dhatwalia (2003), Khagta & Gautam (2016), Kumari & Gautam (2014) , Kumari et al. (2022) and Samkaria et al. (2010). Robinson & Chen (1969) reported 2n = 10 for *M. euphorbiae*, *M. geronii, M. kickapoo, M. lomitoni, M. maintobans* and *M. pallidum*. Blackman (1980) also reported 2n = 10 in other species of *Macrosiphum* such as *M. amygdaloides, M. californicum, M. funestum* and *M. stillariae*. Chen & Zhang (1985b), Robinson & Chen (1969) and Steffen (1968) have reported variation in the genus *Macrosiphum.* The chromosome number 2n = 14 reported here for *R. perosum* is in conformity with earlier reports by Basu (1989) and Kar et al. (1990).In *Sitobion*, the diploid chromosome numbers of 11, 12, 16, 18, and 20 have been reported earlier by Blackman (1980), Chen & Zhang (1985a, b), Dutta & Gautam (1993), Hales et al. (1990) and Robinson & Chen (1969). In *S. rosaeiformis,* the diploid chromosome number of 18 reported here is in conformity with the earlier reports of Dutta & Gautam (1993) and Kumari & Gautam (2014). Gautam & Dutta (1994) have however, reported 2n = 14 and 18 in *S. rosaeiformis*.

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Declaration

The authors have no conflict of interest.

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RESEARCH ARTICLE

A PILOT STUDY ON ANALYSIS OF TYROSINE HYDROXYLASE GENETIC VARIANTS AND ESSENTIAL HYPERTENSION IN CHANDIGARH SAMPLE SET

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SUMMARY Complex condition like Essential hypertension (EH) is common in population still too far in understanding. Catecholamines (CA) are neurotransmitters which are commonly known for their fight and flight response and are the central point to change body's physiology in terms of body temperature, blood pressure, blood glucose level under acute stress. Tyrosine hydroxylase (TH) is the rate limiting enzyme in the CA synthesis pathway and has crucial role in its regulation and yield. Genetic variants of TH have functional implications on gene's expression and found to be strongly associated with nervous disorders. Most studies have explored the role of these variants in nervous disorders worldwide but at national level, their role in EH are highly limited. The present study is focused on the prevalence of TH functional polymorphisms namely, rs10770140, rs10770141 and rs6356 among individuals with and without EH, in a double-blind case-control study design using PCR-RFLP genotyping method and statistical analysis.

Keywords: Sympathetic nervous system, essential hypertension, tyrosine hydroxylase, polymorphism, Indian population.

INTRODUCTION

Essential hypertension (EH) is a condition of high blood pressure without any secondary cause, like thyroid, cardiovascular disorder or renal malfunction (Carretero & Oparil 2000). It falls in the category of complex phenotype where both genetic and environmental factors have unified effect. Sympathetic nervous system (SNS) is a branch of autonomic nervous system with major role in maintaining the homeostasis of body, gives fight and flight response when body is under acute stress. Under stress, there is an increase in body temperature, blood pressure, blood glucose level in order to deal with the unfavourable condition (De Quattro & Feng 2002). SNS works under the influence of neurotransmitters like acetylcholine and CA, among which CAs are secreted at post-ganglionic synapses and by chromaffin cells of adrenal medulla which act as modified sympathetic ganglion. These include dopamine, epinephrine and norepinephrine. Previous studies have shown patients with primary/essential hypertension to have a high level

of catecholamine (De Quattro & Feng 2002). Tyrosine hydroxylase (TH) is the rate limiting enzyme in the catecholamine synthesis pathway and has crucial role in its regulation and yield (Daubner et al. 2011, Rao et al. 2007). Several studies have reported that increased expression of TH gene is associated with increase in synthesis of CAs which further led to increase in blood pressure (Hunt 2007, Rao et al. 2007, Zhang et al. 2004). Population studies have shown that multiple genetic variants exist in this gene which are associated with EH. Most common val/met substitution (rs6356) present in exon 2 is located in the regulatory domain of the enzyme and has been widely studied in neural disorders (Cellorio et al. 2012, Cho et al. 2009). A promoter haplotype reported earlier to have significant effect on the rate of TH gene expression, on further validation studies revealed 2 single nucleotide polymorphisms (SNPs), rs10770140 and rs10770141 with relevant functional effects (Rao et al. 2007). On PubMed search for rs6356, we found only 2 papers from India, which give us allelic frequency of val/met substitution in context with schizophrenia and in individuals with high altitude acclimatization (Srivastava et al. 2010, Tomar et al. 2015). But for rs10770140 (A-581G) and rs 10770141(C-824T) no such data were available from India. The objective of our study was to screen these SNPs in the sample set to establish their allele frequencies and investigate their role in hypertension.

MATERIALS AND METHODS

Blood samples were collected from 198 individuals with the help of clinicians from Bharat Vikas Parishad Charitable Medical Centre, Sector 24, Chandigarh and Bhai Ghanaiya Ji Institute of Health, Panjab University, Sector 14, Chandigarh from 2011 to 2016. All the participants were residents of Chandigarh region, either by birth or for the last 15–20 y. The study was approved by Institutional Ethics Committee (IEC) of Panjab University and all the participants provided their informed written consent for participation voluntarily. The criteria for hypertension included participants with systolic blood pressure (SBP) ≥ 140/diastolic blood pressure (DBP) ≥ 90mm hg or previously diagnosed as hypertensive by physician and/or on regular medication for hypertension whereas individuals with secondary hypertension were excluded from the study. Healthy controls included those with normal blood pressure (SBP < 120/DBP < 80mm hg), none with cardiovascular, renal or endocrine malfunction and were not on any anti-hypertensive medication. To study the base line allelic frequency of rs10770140 (A-581G) and rs10770141 (C-824T), a total of 121 healthy control samples were screened and for association analysis, only age matched control samples were included. DNA isolation was done using conventional phenol chloroform method and PCR-RFLP was used for genotyping (Table 1). Allelic and genotypic frequencies were calculated and sample sets were checked for Hardy-Weinberg Equilibrium (HWE) using HWE calculator (http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html). Allelic and genotypic

TABLE 1: PCR-RFLP details of analyzed SNPs.

SNP (rs ID)	Primer sequence	PCR Programme	No. of cycles	Restriction enzyme	Incubation temp.	Cut pattern
A-581G	F:5' GCGTGGCGTCTCCTTAGA	I:95°C, 7m ; D:94°C, 20s ;	35	BtgI	37°C/ON	G: 87, 73
rs10770140	R:5' GAGTGCCATCTGCCCACA*	A: 55°C, 30s ; E: 68°C, 20s ;				A: 160
		FE: 72°C, 5m				
C-824T	F:5'CTGGTGGGGTAGAGGAGAAA	I:95°C, 7m ; D:94°C, 20s ;	35	DraI	37°C/ON	T: 80, 75
rs10770141	R:5' ACCCCACTCAGCCCCTCTAT *	A: 58°C, 30s ; E: 68°C, 20s ;				C: 155
		FE: 72°C, 5m				
Val/ Met	F:5' ATCCCCTGCCTCTGTGTGCCAT	I:95°C, 5m ; D:95°C, 20s ;	30	NlaIII	37°C/ON	A:170,143,91
rs6356	R:5'TCAGGAACTCAGCCCACACAGC **	A: 66°C, 20s ; E: 72°C, 30s ;				G: 303, 91
		FE: 72°C,7m				

Primer sequences were taken from literature.*' (Zhang et al. 2010); **' (De Quattro & Feng 2002); ON, Over-night.

TABLE 2: Allelic and genotypic profiles of SNPs analyzed.

SNP	Samples	Type	No. of samples	Average Age	MAF	Allelic (p vs q)			pp+ vs pp-			qq+ vs qq-		
						χ^2(p)	OR (95% CI)	p-value	χ^2(p)	OR (95% CI)	p-value	χ^2(p)	OR (95% CI)	p-value
rs10770140	Total	HTN	77	42.29 ± 6.15	0.16	3.38	0.59	0.07	2.96	0.55	0.09	1.10	0.46	0.30
rs10770141		NTN	62	45.17 ± 8.57	0.27	(0.07)	(0.33-1.04)		(0.09)	[0.27-1.09]		(0.29)	(0.11-2.02)	
	Males	HTN	49	41.3 ± 6.31	0.15	4.1	0.48	0.04*	4.25	0.39	0.04*	0.68	0.47	0.42
		NTN	36	46.16 ± 8.7	0.27	(0.04*)	(0.23-0.98)		(0.04*)	(0.16-0.96)		(0.4-1)	(0.07-2.96)	
	Females	HTN	28	44 ± 5.55	0.18	NS	NS	NS	NS	NS	NS	NS	NS	NS
		NTN	26	43.8 ± 8.3	0.19									
rs6356	Total	HTN	76	42.28 ± 6.19	0.47	0.83	0.93	0.77	0.98	1.46	0.32	2.26	0.55	0.14
		NTN	58	45.69 ± 8.6	0.49	(0.77)	(0.57-1.5)		(0.32)	(0.69-3.11)		(0.13)	(0.25-1.2)	
	Males	HTN	48	41.29 ± 6.38	0.44	0.23	0.86	0.63	0.49	1.11	0.82	1.11	0.58	0.29
		NTN	35	46.34 ± 8.76	0.48	(0.63)	(0.46-1.6)		(0.82)	(0.43-2.87)		(0.29)	(0.2-1.6)	
	Females	HTN	28	44 ± 5.55	0.51	0.32	1.07	0.86	1.9	2.45	0.17	1.13	0.51	0.29
		NTN	23	44.69 ± 8.46	0.50	(0.86)	(0.49-2.3)		(0.17)	(0.67-8.93)		(0.29)	(0.15-1.77)	

*, Statistically significant; NS, Non-significant; rs10770140 and rs10770141 are compiled in one row because they were in complete linkage disequilibrium. CI, Confidence interval; MAF, Minor allele frequency; OR, Odds ratio.

frequencies between hypertensive (HTN) and normotensive (NTN) were compared using r*c contingency Table (http://www.physics. csbsju. edu/stats/contingency_NROW_NCOLUMN_form .html). Additionally, frequency distribution was also compared between males and females to analyze any gender bias. Odds ratio with 95% confidence interval was used to find the association between genetic variant and EH by using MedCalc software (https://www.medcalc.org/calc/odds ratio.php).

OBSERVATIONS

A total of 121 healthy NTN individuals and 77 HTN individuals were recruited as per inclusion and exclusion criterion after obtaining the written informed consent from each of the participants and IEC approval for the study. DNA was successfully isolated from each of the blood sample and stored at 4° C till further usage. Genotyping data was statistically analyzed for each of the TH variants and result is presented variant-wise in the following section:

rs10770140 (A-581G) and rs10770141 (C-824T)

Allelic and genotypic data of studied sample set is presented in Table 2. A total of 121 healthy NTN samples were successfully genotyped to reveal the base line frequency of G-581 and T-824 as 0.22. Similar to other studies (Hunt 2007, Rao et al. 2008, Sadahiro et al. 2009) we also found these 2 SNPs in complete linkage disequilibrium. Further segregation of these samples on the basis of age (NTN = 35–65 y, HTN = 30–50 y) for association analysis, reduced the control sample number (NTN = 62). All the NTN and HTN samples were in agreement with HWE. Allelic frequency between HTN and NTN were found to be slightly different (0.16 and 0.27 respectively) which did not amount to statistical significance.

rs6356 (G > A)

Val/Met polymorphism was screened in 62 NTN and 77 HTN but only 58 NTN and 76 HTN could be successfully genotyped. Base line frequency of this variant was 0.49 and no significant statistical difference was observed between NTN and HTN frequencies (Table 2).

DISCUSSION

TH variant rs10770141 (C-824T) did not show any correlation with chronic fatigue syndrome (CFS) and personality disorders (Fukuda et al. 2013, Sadahiro et al. 2010), but a correlation of major allele, C was found with low IQ among schizophrenic patients (Horiguchi et al. 2014). Besides the genotype, promoter haplotypes of TH specially, haplotype 2 (TGGG) i.e. -824T, G-801, -581G and G-494 has shown considerable role in increasing both norepinephrine excretion and blood pressure during stress (Rao et al. 2007, 2008). This was further validated through luciferase reporter plasmid assay where the presence of minor alleles, -581G and -824T, increased the gene expression (Zhang et al. 2010). The study also revealed that -581 and -824 form binding motifs for SP1, AP2, EGR1 and MEF2, SRY, FOXD1 transcription factors (TF) respectively and the presence of minor alleles provide higher strength for binding these TFs. The present study for the first time reports the

frequency of -581G and -824T as 0.22 among the studied sample set from North India and the presence of linkage disequilibrium as similar to other studies (Rao et al. 2008, Zhang et al. 2010). Allelic and genotypic association analysis showed only marginal difference which was not statistically significant. However, gender-based sample segregation showed marginal difference (p value = 0.04, Table 2) among HTN males and NTN males, though female samples did not show any such trend. This marginal difference could be attributed to the fact that -824 also happens to be the binding site for SRY whose different binding strength is known to influence gene expression in males (Zhang et al. 2010). However, further validation studies are warranted to make clear vision about this fact. Further, genotype TT at -824 was associated with high mean SBP (Nielsen et al. 2010), but surprisingly we found higher TT base line frequency in NTN (0.08) rather than HTN (0.039). Statistical analysis including chi-square test and odds ratio have also not shown any statistically significant difference between total HTN and NTN samples.

Another most commonly studied val/met polymorphism is a missense variant, rs6356 whose effects on structure and function of enzyme, TH are not yet completely understood (Cellorio et al. 2012, Ishiguro et al. 1998). Some studies have reported significant association of rs6356 with schizophrenia and restless legs syndrome in female schizophrenia patients (Cho et al. 2009, Srivastava et al. 2010). Our study did not find any convincing difference between the NTN and HTN groups. Nor did the gender-based analysis showed any difference.

The present study reports for the first time, the actual base line frequency of genetic variants, rs10770140 and rs10770141 of TH from India and represents a small-scale investigation into the allelic and genotypic frequencies for the multiple genetic variants of TH among HTN and NTN individuals. Though our study did not find any significant difference between the NTN and HTN groups, it provides the basic frequency information for A-581G and C-824T among Indian population, which could help further studies. We are aware that sample set in this study is too small to comment on association with EH but this data will definitely provide the basis for futuristic large-scale exploration.

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Declaration

The authors have no conflict of interest.

Authors' contributions

PG was involved in the processing of samples, data analyses and drafting of manuscript. The study design was conceived by SC. Supervision of the study as well as correction of the analysis and manuscript was done by SC.

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RESEARCH ARTICLE

A STUDY ON THE GENOTOXIC EFFECT OF CHEWING LEAF TOBACCO ON MOUSE CHROMOSOMES

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SUMMARY In the present study, genotoxic effects of different concentrations of powdered tobacco were investigated in in vivo Swiss albino mice. The chromosomal aberration assay was carried out. Anticancer drug, cyclophosphamide (CP, 50 mg/kg b.w), was used as a positive control agent and distilled water formed the negative control. The doses chosen were, 5, 10 and 20% in distilled water. Different concentrations of crude tobacco were administered orally for 14 consecutive days. The positive mutagen CP was administered only once. Bone marrow chromosome preparations were made at 24, 48, and 72 h after the last administration of crude extract.

Tobacco induced significant chromosomal aberrations (CA) at all doses and time intervals as compared to the negative control group. The effect was both dose and time dependent. The yield of CA was highest at the 24 h time interval and there was a slight recovery from the genotoxic effect at later time intervals. Present results indicate the chromosome damage of crude tobacco in the mouse system.

INTRODUCTION

Tobacco (*Nicotiana tabacum*) in different forms has been used by Indians since time immemorial. Chewing of tobacco is an added risk in the population that is already overburdened with disease. Smokeless tobacco use is widely prevalent among women in southeast Asia and is gaining popularity across the world as a safe alternative to smoking. It is important, therefore, to explore the harmful consequences of this exposure. Research is scanty on the genotoxicity of chewing tobacco.There are reports on the carcinogenic effects of tobacco and probably the genotoxicity is the preliminary event of tobacco carcinogenesis.

Tobacco is used as a narcotic, a hallucinogenic agent, a pain reliever and a pesticide. As such, it has been employed in a wide range of rites and rituals from ancient times. Human societies have used it for both medical and recreational purposes and easily assimilated into a wide variety of cultures (Jassbi et al. 2017). Nicotine is the main addictive substance making people crave for it (Garg et al. 2024).

Tobacco is one of the major economically important commodities whose demand has been increasing in every part of the world. Though it is connected with human cancer manifestations, the smokeless tobacco (ST) product consumption rate is also on the rise. The middle-aged and elderly males and females use more smokeless tobacco. On one side, health ministries of all the countries are stringent on the use of tobacco by imposing tobacco control legislations. On the other, the tobacco industry is trying to attract more people towards the newer products like e-cigarettes and nicotine chewing gum. The world of commerce certainly will not ban tobacco; instead, they are now thinking of coming out with customer-friendly products (Garg et al. 2024).

The studies have shown that tobacco and its components, such as nicotine, can cause genotoxic effects and chromosomal aberrations in various organisms. Since tobacco products and second hand smoke include numerous chemicals that harm DNA, people who use tobacco products and are frequently around environmental tobacco smoke are more likely to develop cancer.

The chromosomal aberration in vivo test in mice evaluates how chemicals affect genetic material and, in turn, how dangerous they are to living things, including normal humans. Information on genotoxic effects at several levels, such as the gene, chromosome, and cellular equipment required for chromosome segregation, is vital to give a comprehensive overview of a chemical's mutagenic and likely carcinogenic potential (Menz et al. 2023).

Tobacco is known to hamper human health (Chen et al. 2017, Kuper et al. 2002, Laldinsangi 2022, Lalruatfela 2019, Padhiary et al. 2021). A large number of studies have been conducted to evaluate the genotoxicity of different forms of tobacco consumed by human beings (Agrawal et al. 2016, Burgaz et al. 2000, Chandirasekar et al. 2019, Khanna et al. 2012, Palaskar & Jindal 2010, Umadevi et al. 2003). Mouse bone marrow cells were genotoxically affected by sadagura which is a form of smokeless product used in Assam (Das et al. 2016). The peripheral blood micronucleus tests and sperm abnormality assays to evaluate the genotoxic effects of chewing leaf tobacco were reported (Ashoka & Mustak 2015). Recently, in a cross sectional study carried out on beedi rollers from Karnataka, showed elevated frequency of micronuclei (Beerappa et al. 2024). The current study aimed to evaluate the effect of long term oral treatment of chewing leaf tobacco on somatic chromosomes of Swiss albino mice (*Mus musculus*) in vivo.

MATERIAL AND METHODS

Swiss albino mice bred and maintained in the animal house, Department of Applied Zoology, Mangalore University, Konaje, Mangalore were used for the experiments. The animal studies were conducted after obtaining the approval from the Institutional Animal Ethical Committee (IAEC) of Mangalore University. Care of the animals and experimental procedures were conducted as per the guidelines of CPCSEA(Committee for the Purpose

of Control and Supervision of Experimentation on Animals), India. Animals were housed in poly-propylene shoe box cages bedded with clean, dry paddy husks and kept in air conditioned room at a temperature of 22 ± 2° C and relative humidity 50 ± 15%. They were fed with a standard pelleted diet (Amrutha feeds, India) and water ad libitum. 8–10 wks old animals of both the sexes with average body weight of 25 ± 2 g were used for the experiments. In each experimental and control group, 5 animals were maintained.

For chromosomal aberration assay, 5%, 10% and 20% chewing leaf tobacco was dosed orally to the animals for 14 d. Three harvesting timings were employed – 24 h, 48 h and 72 h. The dried leaf tobacco was made into fine powder. Then sieved using muslin cloth and ground using mortar and pestle. Oral gavaging was carried out using a glass 1 ml syringe fitted with a needle. This needle was fitted with a 1inch long narrow tube.

Bone marrow chromosomes were analyzed from the cells arrested at the metaphase stage. To obtain a well spread metaphase chromosome, treatment with colchicine and hypotonic solution is very essential. Colchicine, an aneugen is a mitotic arrestant; which inhibits the spindle fiber formation thereby arresting the mitosis at metaphase stage. The hypotonic solution causes the swelling of cells thereby facilitating the spreading of chromosomes (Tjio & Whang 1965).

The animals were injected with 0.025% colchicine (0.2–0.3 ml) intraperitoneally. After 75–90 min, they were killed by cervical dislocation and the marrow cells from femur and tibia were flushed with 0.56% KCl, incubated at room temperature for 20 min. The suspension was centrifuged, and the pellet was dispersed in 1:3 acetic-methanol for 1 h and centrifuged. The pellet was resuspended in acetic-methanol (1:3), incubated for 10 min and centrifuged. This cycle was repeated thrice. An appropriate amount of final suspension was dropped on pre-chilled slides, flame dried and stained with Giemsa.

20–25 drops of stock Giemsa stain was taken in a Coplin jar and diluted with 30–35 ml of 0.01% Triton buffered water (pH 6.8). The slides were stained for 20 min. The excess stain was washed with a phosphate buffer and air-dried. (Note: a good general rule for stain dilution versus staining time is that if dilution is 1:20, stain for 20 min, if 1:30, stain for 30 min and so forth. However, a series of stain dilutions and staining times should be tried to determine the best dilution time for each batch of stock stain).One hundred well spread metaphases were examined per animal (n = 5). The classification of aberrations was done as per the criteria of Savage (1976) and cells were scored using BX 50 Olympus microscope.

OBSERVATIONS

The results of chromosomal aberration assay in mitotic chromosomes of bone marrow cells of *M. musculus* treated with 3 doses (5, 10 and 20%) of tobacco are presented in Tables 1–3.

The tested doses of chewing leaf tobacco

TABLE 1: Percentage frequency of chromosomal aberrations induced by chewing leaf tobacco in bone marrow cells of Swiss albino mice at 24 h.

Treatment	Mean MI ± SEM[#]	GS	BS	EXS	RS	FS	MA	CF	CS	CA	St & Pul	Total % ± SEM[##]
Control	4.22 ± 0.35	0.80 ± 0.37	0.40 ± 0.25	0.00	0.20 ± 0.20	0.20 ± 0.20	0.00	0.00	0.00	0.00	0.00	1.60 ± 0.40
5%Tobacco	3.32 ± 0.16	1.20 ± 0.37	1.00 ± 0.55	2.20 ± 0.86	3.40 ± 0.93	0.60 ± 0.60	0.20 ± 0.20	2.60 ± 1.21	3.00 ± 1.48	2.20 ± 1.20	0.20 ± 0.20	16.60 ± 2.73
10%Tobacco	1.04 ± 0.22 ***	2.00 ± 0.71	1.20 ± 0.49	1.40 ± 0.40	4.00 ± 1.55	0.00	0.00	5.60 ± 1.12**	0.40 ± 0.25	18.20 ± 5.03***	1.80 ± 1.11	34.60 ± 4.96*
20%Tobacco	1.70 ± 0.27 ***	17.00 ± 3.08 ***	4.40 ± 1.50*	14.80 ± 4.25***	15.40 ±4 .09***	0.00	0.00	5.00 ± 1.30*	13.40 ± 6.19 *	13.00 ± 1.70**	8.00 ± 1.14***	91.00 ± 14.89***
CP-(50mg/kg bw)	2.0 ± 0.12 ***	10.60 ± 1.54**	7.80 ± 1.16 ***	7.20 ± 0.86	3.00 ± 0.84	10.80 ± 1.36***	3.00 ± 0.89***	9.00 ± 1.52***	1.60 ± 1.36	0.60 ± 0.40	6.80 ± 1.83***	60.40 ± 3.09 ***

MI - Mitotic index, GS - Gaps, BS - Breaks, EXS - Exchanges, RS - Rings, FS - Fragments, MA - Multiple aberrations, CF- Centric fusion, CS - Centromeric separation, CA - Centromeric associations, St & Pul -Stickiness and pulverization, One-way ANOVA test; n = 5* - P < 0.05. ** - P < 0.01. *** - P < 0.001 (Dunnett's multiple comparison test). # - Mitotic index from 2000 cells/animal, ## - From 100 metaphases/animal.

TABLE 2: Percentage frequency of chromosomal aberrations induced by chewing leaf tobacco in bone marrow cells of Swiss albino mice at 48 h.

Treatment	Mean MI ± SEM[#]	GS	BS	EXS	RS	FS	MA	CF	CS	CA	St & Pul	Total % ± SEM[##]
Control	4.31 ± 0.32	1.00 ± 0.45	0.40 ± 0.25	0.00	0.60 ± 0.40	0.20 ± 0.20	0.00	0.60 ± 0.40	0.00	0.00	0.60 ± 0.40	3.40 ± 0.68
5%Tobacco	3.82 ± 0.11 **	0.60 ± 0.40	1.20 ± 0.74	2.40 ± 1.75	2.80 ± 0.86	1.00 ± 0.78	0.40 ± 0.40	1.80 ± 0.97	0.00	3.00 ± 0.89	0.20 ± 0.20	13.40 ± 2.38
10%Tobacco	2.65 ± 0.13 ***	2.40 ± 0.68	1.60 ± 0.68	6.00 ± 1.18**	8.80 ± 1.39 ***	1.80 ± 0.58	0.60 ± 0.40	5.20 ± 0.86*	1.40 ± 0.51	4.00 ± 1.00	2.40 ± 0.68	34.20 ± 3.89 ***
20%Tobacco	2.39 ± 0.21***	7.00 ± 1.52 **	1.40 ± 0.93	8.00 ± 1.70 ***	6.20 ± 2.06 *	0.20 ± 0.20	0.00	5.00 ± 1.38 *	4.40 ± 1.17**	20.00 ± 4.81***	0.20 ± 0.20	52.40 ± 9.49 ***
CP-(50mg/kg bw)	2.44 ± 0.06***	7.80 ± 1.53***	6.80 ± 1.53 ***	4.60 ± 0.93*	2.40 ± 0.51	6.60 ± 1.33***	3.00 ± 0.89***	8.20 ± 1.32***	1.60 ± 1.36	0.60 ± 0.40	8.60 ±1.33***	50.20 ± 1.36***

MI - Mitotic index, GS - Gaps, BS - Breaks, EXS - Exchanges, RS - Rings, FS - Fragments, MA - Multiple aberrations, CF- Centric fusion, CS - Centromeric separation, CA - Centromeric associations, St & Pul - Stickiness and pulverization, One-way ANOVA test; n = 5* - P < 0.05. ** - P < 0.01. *** - P < 0.001 (Dunnett's multiple comparison test). # - Mitotic index from 2000 cells/animal, ## - From 100 metaphases/animal.

TABLE 3: Percentage frequency of chromosomal aberrations induced by chewing leaf tobacco in bone marrow cells of Swiss albino mice and controls at 72 h.

Treatment	Mean MI ± SEM#	GS	BS	EXS	RS	FS	MA	CF	CS	CA	St & Pul	Total % ± SEM ##
Control	4.30 ± 0.18	1.60 ± 0.25	0.80 ± 0.37	0.00	0.20 ± 0.20	0.40 ± 0.25	0.00	0.00	0.40 ± 0.40	0.00	0.20 ± 0.20	3.60 ± 1.03
5%Tobacco	3.94 ± 0.19	1.00 ± 0.32	1.40 ± 0.51	2.20 ± 0.58	2.00 ± 0.71	0.20 ± 0.20	0.00 ± 0.00	1.20 ± 0.58	0.80 ± 0.37	1.60 ± 0.51	0.00 ± 0.00	10.40 ± 0.51
10%Tobacco	3.82 ± 0.11	2.40 ± 1.60	0.20 ± 0.20	5.80 ± 2.06*	6.60 ± 1.69 **	1.60 ± 0.60	0.20 ± 0.20	12.20 ± 2.75***	0.00	5.60 ± 1.63	9.20 ± 1.91***	43.80 ± 5.81 ***
20%Tobacco	2.49 ± 0.16 ***	1.60 ± 0.68	1.40 ± 0.51	3.00 ± 0.89	4.00 ± 1.55	3.00 ± 1.30	0.00	4.00 ± 0.71	0.80 ± 0.58	7.20 ± 3.14*	8.80 ± 1.28 ***	33.80 ± 4.31 ***
CP-(50mg/kg bw)	2.73 ± 0.19 ***	7.20 ± 1.16***	4.60 ± 0.98***	7.40 ± 1.81**	1.80 ± 0.58	6.40 ± 1.50***	3.00 ± 0.89***	0.80 ± 0.37	1.60 ± 1.36	0.60 ± 0.40	3.60 ± 0.68	37.00 ± 3.38***

MI - Mitotic index, GS - Gaps, BS - Breaks, EXS - Exchanges, RS - Rings, FS - Fragments, MA - Multiple aberrations, CF - Centric fusion, CS - Centromeric separation, CA - Centromeric associations, St & Pul - Stickiness and pulverization, One-way ANOVA test; n = 5* - $P < 0.05$. ** - $P < 0.01$. *** - $P < 0.001$ (Dunnett's multiple comparison test). # - Mitotic index from 2000 cells/animal, ## - From 100 metaphases/animal.

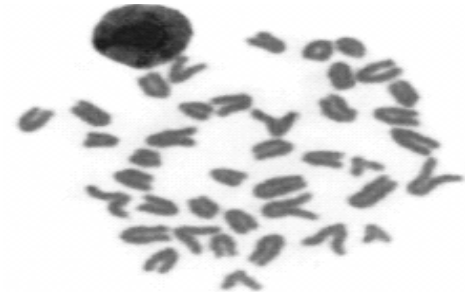
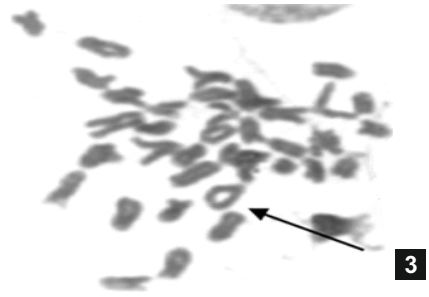
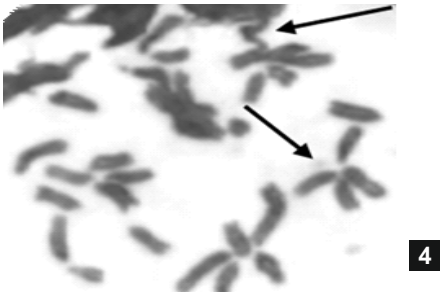
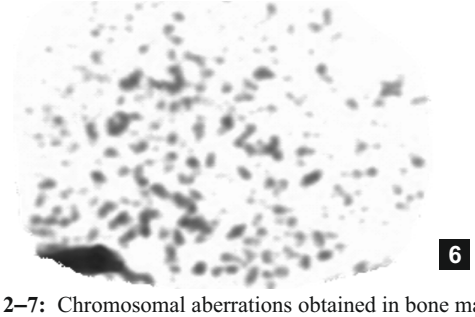


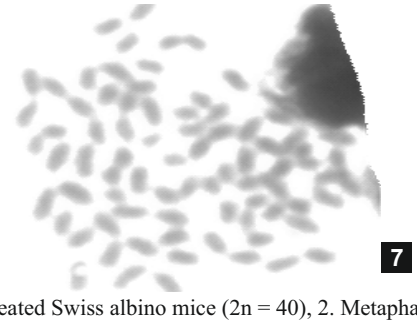
Fig. 1: Bone marrow chromosomal metaphase spread of Swiss albino mouse showing acrocentric chromosomes (2n = 40).

induced various types of aberrations such as gaps, breaks, exchanges, fragments, multiple aberrations, centromeric separation, centromeric associations. It also induced unconventional aberrations like stickiness and pulverization (Figs 1–7). Significant overall abnormalities were produced by all 3 tobacco dosages in a dose-dependent manner. In 24 h harvesting (Table 1), the mean chromosomal









Figs 2–7: Chromosomal aberrations obtained in bone marrow of tobacco treated Swiss albino mice (2n = 40), 2. Metaphase showing breakage of a chromosome (arrow), 3. Metaphase showing a ring (arrow), 4. Metaphase plate showing centromeric association (arrow), 5. Metaphase plate showing a gap (arrow), 6. Metaphase plate showing pulverization.7. Metaphase plate showing centromeric separation.

aberrations in chewing leaf tobacco group ranged between 16.60 ± 2.73 and 91.00 ±14.88 whereas, in the controls this value was 1.60 ± 0.44. Such a percentage represents a 10.37 to 56.87-fold increase as compared with that value of the untreated control group in bone marrow cells. Though there appeared progressive recovery from damages at 48 and 72 h post-sampling time (Tables 2, 3), total chromosomal aberrations were statistically significant from untreated control groups. As far as individual aberrations were concerned, high frequency of centromeric associations occurred in the treated groups.

DISCUSSION

The present study was carried out to evaluate the genotoxicity of aqueous extract of chewing leaf tobacco. Here, we employed 14 d oral treatment. Thus, there are chances that some chemical components must have been bioaccumulated to give the additive effect.

The chromosomal aberration test measures the extent of hereditary injury caused by physical or chemical substances of economic importance. The health surveillance programme use different genotoxicity biomarkers of which in vivo chromosomal aberration test stands as a paramount one. The published literature on the genotoxicity of tobacco smoke inhaled by humans has been implicated for the adverse health effects (Addissouky et al. 2024, Fanoudi et al. 2024, Felix & Ntarisa 2024).

It is commonly recognized that uniting 2 damaged chromosomes can cause chromosome aberration; however, if the break is rejoined (restituted), the original form is restored, eliminating any apparent aberration. A noticeable aberration is often created when the broken ends of this break reunite with the broken ends of other chromosomes. Additionally, the break could stay open to create a visual break (Varga & Aplan 2005).

There are more studies using onion root systems establishing the genotoxic nature of tobacco. In the present study, the given doses of leaf tobacco to Swiss albino mice resulted in many types of chromosomal aberrations in the bone marrow cells. Mojidra et al. (2009) also obtained similar results in their studies where they treated animals with pan masala plain and gutkha (Mojidra et al. 2009). Using score of chromosome/chromatid breaks, Sundaramoorthy et al. (2013) reported significantly higher frequency of chromosome aberrations in lymphocyte cultures from beedi workers in V ellore region (6.85 ± 2.67) than in controls (1.89 ± 1.31). In a similar study done on beedi rollers occupationally exposed to beedi tobacco dust in Jabalpur expressed significantly ($P<0.05$) increased chromosome aberration in terms of percentage (3.0 ± 0.63 and 3.7 ± 0.39 in 30–35 y and 60–65 y of age groups when compared to age matched controls 1.3 ± 0.32 and 1.8 ± 0.24 respectively) (Gautam et al. 2015).

In the present study, the increased percentage of total chromosomal aberrations was due to more centromeric association type. The reports of such a type of chromosomal aberration are limited. Only a few animal studies have reported centromeric

associations (Dkhil et al. 2011).

In our study, we observed the effect of leaf tobacco on the proliferation of bone marrow cells of Swiss albino mice. The higher concentration of 20% tobacco did not result in substantial decrease in mitotic index at 24 h sampling when compared to that of the lesser dose i.e. 10%. All the doses suppressed the mitotic index as compared to the control. In an in vitro study, Yadav & Thakur (2000) reported significantly higher mitotic index value in the bidi smokers.

The environment has a significant impact on the condition of the chromosomes, which are finely balanced systems in metabolically active cells. This delicately balanced system can be altered by any externally delivered drug. Chromosome structural alterations are the result of the DNA component modifications. Usually, only cells that are replicating their DNA create chromosome abnormalities (Brewen & Preston 1973) and the replication of damaged DNA in cells causes mutations of all kinds, including minor deletions, base substitutions, and genome arrangements, to continuously accumulate in somatic cells (Panier et al. 2024). Higher fre-quency of stickiness and pulverization was observed in chewing leaf tobacco treated groups which was also dose dependent. Although they are considered as unconventional aberrations by Savage (1976), they strongly imply the damage caused at chromosome level by the leaf tobacco treatment. The chromosome stickiness and clumping is due to the inhibition of DNA topoisomerase II and the compounds which induce topoisomerase inhibition were found to have more genotoxic effects (Dutta & Mandal 2024, McClendon & Osheroff 2007).

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