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AN IN VITRO RESTORATIVE APPROACH FOR TRISOMY AND MONOSOMY USING GARLIC EXTRACT

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ABSTRACT

Aim: With the discovery in 1956 that the correct chromosome number in humans is 46, several major chromosomal syndromes with altered numbers of chromosomes were reported, i.e. Down syndrome (DS; trisomy 21), Turner syndrome (TS; 45, X) and Klinefelter syndrome (47, XXY). DS is the commonest autosomal chromosomal abnormality in the newborns and its incidence is 1 in 826 live births. It has been well established that chromosome abnormalities contribute significantly to genetic disease resulting in reproductive loss, infertility, still births, congenital anomalies, mental retardation, abnormal sexual development, and pathogenesis of malignancy. 50% of miscarriages and reproductive loss is contributed by autosomal trisomies in Ist trimester of fetal gestation. Whereas monosomic condition in fetal development leads to spontaneous abortion and so far only 1% survival rate has been reported and the causes of which are unknown. In this study we have taken an in vitro approach to study the response of amniocytes and chorion villi samples on treatment with garlic extract (GE). Main Methods: Our study included 120 samples obtained from amniotic fluid and chrionic villi sampling for studying trisomy 21 and monosomy of X Chromosome . GE (12mg/ml) was added to fibroblast culture, incubated and effect of the same was analyzed by inter phase FISH. Key Findings: Incubation of trisomy and monosomy samples with GE (12mg/ml) has revealed normal chromosomal pattern and is statistically significant ($P<0.0001$). Significance: Garlic extract seems to have a significant effect on spindle fiber formation and cell division.

KEYWORDS: Aneuploidy, Trisomy, Monosomy, Allium sativum.

INTRODUCTION

The primary information of human numerical aberration of chromosomes was the detection of trisomy 21 Down syndrome (DS) in 1959. In India, the reported incidence of DS is around 1 in 1250 live births. In 95% of DS, the extra 21 is in a free state because of the parental meiotic non-disjunction phenomenon during gametogenesis. 90 to 95% of the trisomy 21 condition in DS is because of the maternal meiotic error. Beginning in the 1980s, several groups initiated DNA polymorphism studies to determine the origin of human aneuploidy. Loss of sex chromosome is due to non-disjunction during gametogenesis is a relatively universal event that contributes to formation of zygotes monosomic for an X- or Y-chromosome as well as zygotes with supernumerary sex chromosomes. Monosomy for the elementary, gene-poor human Y chromosome is not compatible with survival, but embryos endowed with a single X chromosome are viable for the early stages of growth and a small percent survive to term and into adulthood. Trisomy of chromosome 13 or Patau Syndrome, trisomy of chromosome 18 or Edward Syndrome and trisomy of chromosome 21 or Down syndrome are compatible with survival.[1] In all cases the presence of an additional chromosome copy results in a multifaceted pathologic phenotype (For instance there are around 72 pathological features linked to trisomy) that regularly sternly has weaken quality of living. The causes for the spectacular effect of the trisomies as well as the molecular machineries underlying the phenotypes are not fully understood.[2] Accordingly, no targeted treatment is existing for patients despite several decades of intense research. But various drugs including Dehydroepiandrosterone (DHEA), an endogenous adrenal steroid showed a lower rate of embryonic aneuploidy but has enormous side effects.[3]

Knowing the side effects of various chemical molecules like genotoxic, proteotoxic, steroids, we emphasized the importance of substances of natural origin, including whole plants or their component. In addition, in Ayurveda diverse procedures are used in an effort to maintain well being in a healthy person and alleviate disorders of the body and brainpower. These materials work on the principles of samanya (homologous) and visesha (opposed) action. Matters possessing homologous characteristics and actions increase the relevant elemental properties of the body while those having antagonistic actions decrease those properties or components. In cases of disease or disparity of dosha (humours), dhatu (tissues) and mala (waste materials), the balanced use of naturally obtainable substances aspires to restore normality. Keeping this in mind an *in vitro* approach was taken to study the effect of garlic (*Allium*

sativum) on trisomy and monosomic samples. Garlic has been used since prehistoric times, as a spice and also known for its remedial properties.

MATERIALS AND METHODS

Cell Culture and Geimsa Trypsin Banding

Amniotic Fluid Culture Set up

15-20 ml of amniotic fluid was collected in sterilized condition, culture set up was done with 5 ml of Amniomax II complete media. Cells were dispensed into a T-25 flask and mixed well, taken care that the amniotic fluid cells/ amniocytes spread evenly on the surface of the flask. 6-7th day flasks were observed for colony growth and attachment and on ninth or tenth day cells were arrested with colchicine solution (10µg/ml) and dissociation was done with 1 X Trypsin/ EDTA. Flasks were gently taped to release maximum nuclei from colonies and harvested with prewarmed hypotonic solution (1% sodium citrate). Nuclei were fixed using 1 ml of cold fixative added by constant stirring. Fixation was repeated twice and cells were dropped onto clean and cold slides stored at 4^{o}C. 20x phase contrast microscopy was used to check for spreading and mitotic index. Slides after air drying were baked at 60°C in incubator overnight and proceeded with Geimsa Trypsin Banding. In Geimsa Trypsin Banding, the metaphase chromosomes were treated with porcine trypsin and stained with GIEMSA, a nuclear stain. Heterochromatic regions, which are rich in Adenine and Thymine (AT-rich) are relatively gene-poor, stained more darkly in G-banding. In contrast, less condensed chromatin rich in Guanine and Cytosine (GC-rich) are more transcriptionally active, incorporated less Giemsa stain, and these regions appeared as light bands in G-banding. Chromosomes from fibroblast cells showed banding in 15-20 sec.

Chorion Villi Culture set up

25-30 mg (approx) of chorionic villi sample was collected in transport media (Amniomax II complete media + penicillin +streptomycin + nystatin). Chorion Villi were treated with 2ml of 5mg/ml of Collagenase II enzyme. Cells were dispensed into a T-25 flask and mixed well, taken care that the cells spread evenly on the surface of the flask. 6-7th day flasks were observed for colony growth and attachment and on ninth or tenth day cells were arrested with colchicine solution (10µg/ml) and dissociation was done with 1 X Trypsin/ EDTA. Flasks were gently taped to release maximum nuclei from colonies and harvested with (1% Sodium Citrate) pre warmed hypotonic solution. Nuclei were fixed using 1 ml of cold fixative added by constant stirring. Fixation was repeated twice and cells were dropped onto clean and cold

slides stored at 4°C. 20x phase contrast microscopy was used to check for spreading and mitotic index Slides after air drying completely were baked at 60°C in incubator overnight and proceeded with Geimsa Trypsin Banding. Geimsa Trypsin Banding was done as described earlier.

Our study is based on 2 sets of culture. In SET 1, when cells attained 75-80% confluency, 12mg/ml GE was added, incubated for 48-72hrs and either conventional karyotyping or FISH was performed. In Set II, cell pellets were retrieved from original amniotic fluid and chorion villi stored sample. Further incubated with 12 mg/ml GE in 5ml amniomax II complete medium for 72 hrs and only FISH was performed.

Fluorescence In Situ Hybridization (FISH)

FISH was performed on uncultured amniocytes and chorion villi to evaluate the aneuploidy status. 0.05% 1X Trypsin EDTA was used to obtain interphase nuclei followed by pre warmed 1% Sodium citrate hypotonic treatment with continuous vortex. Fixation of cells were done by using 1ml chilled fixative (Carnoy's Fixative) and repeated twice. Cell pellet was spread on to the slide and labeled with a pencil. Slides were aged in pre warmed 2XSSC followed by dehydration in 70%, 85%, 100% alcohol series 2 min each at room temperature and air dried. 10µl of probe was added on to small selected area of hybridization with coverslip on and sealed with rubber cement. Denaturation of the slide was done at 75ºC for 5 minute and hybridized overnight at 37ºC in Hybrite. On day 2, post hybridization, washes were done in 0.4XSSC/0.3%NP40 at 72 ˚C for 2 min, with slight agitation in between. Followed by room temperature wash in 2X SSC/0.1%NP40, Counter staining was done by adding 10 µl of DAPI on to the area of hybridization. 12X12mm cover slip was put on and slides stored at 4ºC until microscopy.

MICROSCOPY

Capturing of the images was done in specific DAPI/FITC/TRITC/SPECTRUM AQUA filter in 100X oil immersion. Clear signals were counted from 100 interphase nuclei. Overlapping cells were not considered in analysis. In cases of abnormality, or if extra signal was seen the counting was increased to 50 cells. Analysis was performed using ISIS from Metasystem. Metaphases were selected for karyotyping using IKAROS, Metasystem.

RESULTS

Our study included 127samples for trisomy 21 and 20 samples for monosomy X. Various herbs were scrutinized such as tulsi, ginger, cinnamon, lodhra. However garlic was found to give the best results. GC-MS analysis of methanol extracted garlic source was used in this study (GE was obtained from Durga Femto Technologies and Research (DFTR), Bangalore. Karnataka, India). Other selected herbs and their corresponding extracts were found to be unsuitable in terms of pH and causes of contamination in the cultured sample. GE has a proven effect on sister chromatid exchange (SCE) and has anti cancerous properties and SCE plays a significant role in recombination. Various concentrations of GE was tried ranging from 6, 12 and 24 mg/ml. GE showed promising results at a concentration of 12 mg/ml. DNA synthesis and replication takes 8-12 hours and our focus was to give a cycle of 72hrs of replication to observe any kind of promising effects in *in vitro* condition.

Our entire study was done with 2 different sets of culture (SET I, SET II) as mentioned in materials and methods using following samples.

1. Trisomy of 21st Chromosome
2. Monosomy of X Chromosome

1. Trisomy of 21st Chromosome

Karyotype and FISH images are showing Trisomy 21 before GE treatment (Fig 1a, b).

In SET I Culture only FISH was performed and 100 nuclei were scored for investigation. Our analysis revealed the normal pattern which is statistically significant ($P<0.0001$) implicating the effect of GE on Sister Chromatid exchange and probable curative effect on recombination during the cell cycle stage. However cells showed +21 status also (Fig 1c). Flasks on 7th day onwards started showing degradation of cells, eventually on 10th day a lot of populated dead cells were observed hence we could not proceed with conventional cytogenetics.

In SET II culture, the normal pattern was observed which is statistically significant ($P<0.0001$) (Fig 1d, Fig1e). The entire picture is suggesting a positive effect of GE on trisomy 21.

2. Monosomy of X Chromosom

Preliminary findings of the sample confirmed Monosomy of X chromosome resulting in classical Turner's Syndrome. The tests were performed using conventional karyotyping and FISH on interphase nuclei (Fig 2 a,b). SET I culture only interphase FISH was done using

DNA probes. 100 nuclei were scored for analysis, normal pattern was observed which is statistically significant (P<0.0001) implicating the effect of GE on Sister Chromatid exchange and probable curative effect on recombination during the cell cycle stage. The cells however showed monosomy X status also (Fig2c). In SET II culture, the normal pattern was observed which is statistically significant (P<0.0001) after scoring 100 nuclei (Fig 2d, Fig 2e).

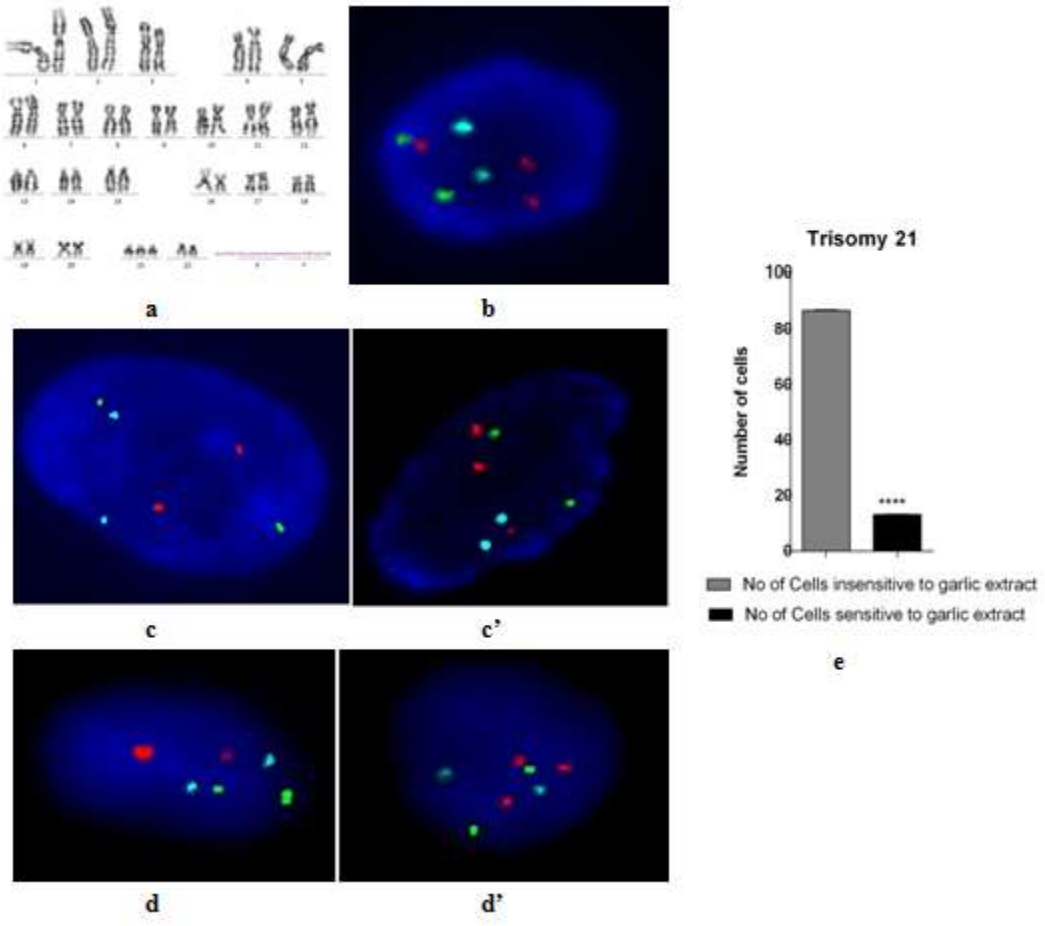

Fig 1 a. Karyotype image showing Trisomy 21 before GE treatment,

b. FISH image of trisomy 21 before GE treatment (3 Red Signals for Trisomy 21(abnormal pattern) 2 Green Signals for Chromosome 13(normal pattern)2 Blue Signals for Chromosome 18 (normal pattern),

c. FISH image of trisomy 21 sample when GE (12mg/ml) added at 70-80% confluency and was incubated for 72 hrs (SET I culture) Normal pattern (2 Red Signals for Trisomy 21).

c' FISH image of trisomy 21 sample when GE (12mg/ml) added at 70-80% confluency and was incubated for 72 hrs (SET I culture). Abnormal pattern (3 Red Signals for Trisomy 21), 2 Green Signals for Chromosome 13 (normal pattern) 2 Blue Signals for Chromosome 18 (normal pattern).

d . FISH image of Trisomy 21 incubated with GE (12mg/ml) for 72 hrs (SET II culture) normal pattern (2 Red Signals for Trisomy 21)

d'. FISH image of Trisomy 21 incubated with GE (12mg/ml) for 72 hrs (SET II culture) abnormal pattern (3 Red Signals for Trisomy 21), 2 Green Signals for Chromosome 13 normal patter, 2 Blue Signals for Chromosome 18 normal pattern

e. Effect of GE (12mg/ml) on Trisomy 21 samples. Values are mean ±SEM for n=100 nuclei per 100 samples. **** p<0.0001 versus number of cells insensitive to garlic extract. It was compared by one way ANOVA followed by Wilcoxon test.

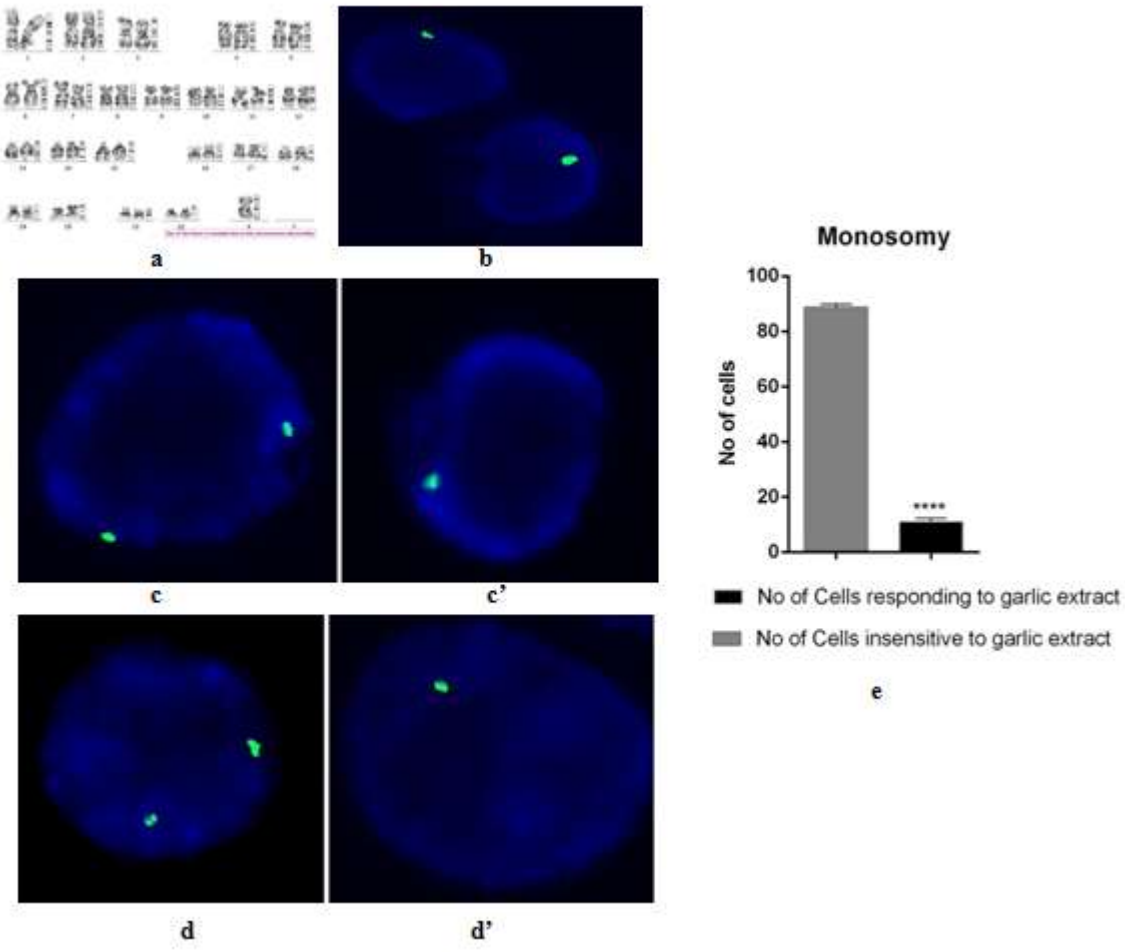

Fig 2. a Karyotype Image for Monosomy X before GE treatment

b. FISH image of Monosomy X before GE treatment 1 Green Signal represents one copy of Chromosome X

c. FISH image showing pattern for X chromosome after GE (12mg/ml) treatment on 70-80% confluent cells(SET I culture) .Normal signal pattern, *2 Green Signal Depicts X chromosome pattern*

c'. FISH image showing pattern for X chromosome after GE (12mg/ml) treatment on 70-80% confluent cells (SET I culture) Abnormal signal pattern for X chromosome *1Green Signal Depicts X chromosome pattern*

d. FISH image showing pattern for X chromosome after GE treatment (SET II culture).Normal signal pattern, *2 Green Signal Depicts X chromosome pattern*

d'. FISH image showing pattern for X chromosome after GE treatment (SET II culture). Abnormal signal pattern for X chromosome *Green Signal Depicts X chromosome pattern*

e. Effect of GE (12mg/ml) on Monosomy samples. Values are mean ±SEM for n=100 nuclei per 20 samples. **** p<0.001 versus number of cells insensitive to garlic extract. It was compared by one way ANOVA followed by Wilcoxon test

DISCUSSION

For centuries, the garlic is used as a medicinal herb. It is having wide range of medicinally important properties like, antimutagenic, anticancer, anti-inflammatory, antihypertensive, antifungal, antidote, hepatoprotective, antimicrobial, hyperglycemic, immunomodulation, antigenotoxic, antimutagenic etc.[4,5,6,7,8,9,10] Studies of the anticarcinogenic effects of garlic on several carcinogens were found to be effective in different ways such as direct inhibition of tumor cell metabolism, inhibition of initiation and promotion phases of carcinogenesis and modulating the post immune response and besides all these garlic acts as a strong antioxidant by its ability to scavenge free radicals.[11] Sulfur rich constituents of garlic such as diallyl sulfide (DAS) and diallyl disulfide (DADS) are known to induce activities of phase II enzymes, which in turn reduce the genotoxicity of several carcinogens.[12]

During the recent years much focus has been given for the search for natural compounds which modulates the drug.[13] Experimentally, garlic and its coupled sulfur constituents are reported to reduce the chemical induced toxicity.[14] Several studies have clearly established the protective effects of various phytonutrients upon drug-induced toxicity and suppress[15] the tumor occurrence in skin, breast uterine, colon, esophagus and lung cancers. This protection is presumed to arise from several mechanisms including enhanced DNA repair.[16] In this study we used the garlic extract. Among the major components of garlic, allicin, DAS, S-allylcystine and other organo-sulfur compounds have the radical scavenging activity and protective effects against chemical induced toxicity.[17] The DAS and related compounds from garlic have inhibitory effects on chemical carcinogenesis and mutagenesis.[18] The most

likely mechanism behind reduction in the rate of Trisomy (Fig 1 c,d,e graph) and mosomy (Fig 2 c,d,e graph) condition can be attributed to the action of free radical scavenging, escalating the action of antioxidant enzymes and inhibit the DNA adducts formation influencing the repair mechanism and modulating several metabolizing enzymes like cytochrome p450 and GST's. Two groups[19,20] studied modulatory effects of garlic extract against the cyclophosphamide induced genotoxicity in human lymphocytes *in vitro* using chromosomal aberrations (CA) and sister chromatid exchanges (SCE) assays method. The results indicated a significant decrease in the frequency of CA and SCE**.** Similarly diallyl trisulfide (DATS) was shown to induce apoptosis in prostate cancer cells involving c-Jun N-terminal kinase and extracellular signal-regulated kinase-mediated phosphorylation of Bcl-2. Human leukemia cell line HL60 when treated with ajoene, a component of garlic, both trypsin- and chymotrypsin-like activities were influenced, cells arrested in G2/M phase and total amount of cytosolic proteasome increased. The garlic-derived compound DADS exerts anti proliferative effects by binding directly to tubulin and disrupting the microtubule assembly, thus arresting cells in mitosis and triggering mitochondria-mediated signaling pathways that lead to apoptosis.

CONCLUSION

We see GE to have diverse effect in fibroblast tissue with ploidy samples and acting to counter the effect of non disjunction, thus producing smaller portion of normal cells as seen in our results. At this point of time more investigation is needed to delineate the down regulation pathways of modulatory actions of extract of garlic and which component of it is exerting effect on aneuploidy in *in vitro*. We have predicted that this finding of ours gives the directions for the future research possibilities for the design and development of garlic extract related modulatory drugs. Such drugs might minimize the side effects caused by the widely used genotoxic, proteotoxic, steroids etc. Modulatory effects of garlic have been demonstrated in animals and some in humans, there is need for better-designed double blind clinical trials to better determine curative value, dosage levels, extent of therapy, and other factor.

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"**Conflict of interest statement"-** The authors declare that there are no conflict of interest.

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