EVALUATION OF SISTER CHROMATID EXCHANGES IN CATTLE REARED IN A RADIO ACTIVE BELT AREA OF SOUTHERN KERALA

By
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THESIS

Submitted in partial fulfilment of the
requirement for the degree

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DECLARATION

I hereby declare that this thesis entitled **EVALUATION OF SISTER CHROMATID EXCHANGES IN CATTLE REARED IN A RADIO ACTIVE BELT AREA OF SOUTHERN KERALA** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled "**EVALUATION OF SISTER CHROMATID EXCHANGES IN CATTLE REARED IN A RADIO ACTIVE BELT AREA OF SOUTHERN KERALA**" is a record of research work done independently by **Sri. C.N. Dinesh.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Introduction

INTRODUCTION

Animal Husbandry is closely interwoven with agriculture and plays an important role in our national economy. In Kerala, the significance of this sector is heightened by its impressive contribution which stands at 10.58 per cent of the Gross Domestic Product (GDP) in 1993-'94.

According to 1987 Livestock Census, the bovine population in Kerala is 3.7 million of which 91 per cent are cattle and the rest buffaloes. This State has succeeded in transforming nearly 60 per cent of the cattle population to crossbreds during the last 25 years by adopting different comprehensive cattle breeding programmes and policies. These achievements have also been reflected on milk production front which increased phenomenally from 2.21 lakh tons per year in 1963-'64 to 22.2 lakh tons in 1994-95.

Though the advances in science and technology have contributed much to the development of human and animal resources, uncontrolled industrialisation and urbanization resulted disturbances in the ecosystem. Untreated industrial effluents and indiscriminate use of various insecticides, pesticides and chemical fertilizers have resulted an environmental system where the living organism is subjected to various toxic agents.

The exposure to radioactive elements and its decay products is now established as a source of various damages to existing living and non-living subjects. The increased application of nuclear energy as a modern tool in many human endeavours raised many embarassing questions concerning the long-term effects of radiation on life.

Cytogenetics is a branch of genetics which correlates the microscopically discernable genetic units to that of its phenotype. Cytogenetic studies have revealed that animals and plants exposed to radiation exhibit genetic damage which in turn leads to various biological changes.

The acute radiation and its hazardous effect on living organisms were explained based on the survey of survivors of atomic bomb explosions in Hiroshima and Nagasaki. However, there is a distinct lack of quantitative data on the real long-term consequences to biological systems exposed to continuous doses of radiation for very long periods of time. Areas with naturally occurring high background radioactivity offer unique opportunity for obtaining this information.

One such geographical area lies along the south-west coastal region of Kerala. Here a stretch of about 55 km with definable geographical landmarks includes the most concentrated distribution of monazite. It is one of the best known of the high background radiation areas in the world,

even upto 250 times greater than the normal background radiation levels of 0.02 mR/hr. The radio activity in these areas is due to the presence of sand admixed with monazite which contains principally thorium 232 and its radioactive decay elements. The area of present study - Chavara panchayat lies in this belt.

All this underline the importance of undertaking research on the effects of high background radiation on genetic constitution of living organisms. The assessment of cattle in this area in terms of sister chromatid exchange (SCE) frequencies are sensitive indices of chromosomal damage. This technique provides simple, reliable and highly sensitive method for detecting mutagenicity and environmental pollution.

It is obvious that a study in this line would help to understand the damage to genetic material by background radioactivity on farm animals. Rightly so, the proposed cattle breeding policy for Kerala underscores the importance of techniques like SCE in assisting the elucidation of drastic environmental, chemical and radiation hazards to livestock. But no work has so far been taken up to evaluate the changes in bovine chromosomes due tc long-term background radiation using cytogenetic techniques. Hence this study is planned with the following objectives.

1. To standardize the technique for assessing SCEs in cattle.

2. Evaluation of SCEs in cattle reared in a radioactive area (Chavara) of southern Kerala.

Review of Literature

REVIEW OF LITERATURE

2.1 Background radiation

Investigations on populations which are subjected to high levels of natural background radiation offer possibilities for evaluating the long-term effects of chronic radiation exposure on life. It has been the fervent hope that investigations on such populations may perhaps lend themselves to critical analysis and thus provide meaningful answers on the effect of chronic radiation exposures on human, animal and plant populations.

One geographical area where large number of living organisms are exposed to high background radiation lies over a stretch of about 55 km along the coastal region of Kerala. This coastal line is characterised by patches of radioactive sand which is rich in radioactive thorium and its decay products.

Ayengar *et al.* (1970) conducted cytological studies on the biological effects of high background radioactivity on plants growing in the monazite bearing areas of Kerala coast and adjoining regions and reported that the incidence of meiotic abnormalities was higher in samples from the radioactive areas as compared to those from the control belt.

Detailed analysis of data revealed the relationship between the external radiation level, internal radio-nuclide content and cytological abnormalities.

Ayengar *et al.* (1971) conducted extensive demographic survey and radiation dosimetric measurements of households and personal in the coastal strip of about 55 kms extending from Thekkumbhagum in Kollam district in the south to Purakkadu in Alleppy district in the north. This study revealed that 24.6 per cent of the households recorded levels greater than five times the normal natural background radiation level, 8.8 per cent of the households received more than 10 times the normal radiation level and 1.1 per cent recorded more than 20 times the normal level. It was also established that as one proceeded from Purakkadu in the north to Thekkumbhagum in the south, the fraction of households in each area receiving greater than 5 times normal background radiation exposure levels gradually increased with the maximum value in Chavara area. Further the percentage of households receiving greater than 10 times the normal levels were also observed to be the highest in Chavara and Thekkumbhagum areas. They worked out the per capita dose for the entire population residing in the region as 297 mR per year which was nearly four times the normal background radiation level.

Dang *et al.* (1992) estimated the daily dietary intake of 232 Th and 238 U by the population living in the high background radiation areas of India (monazite area) where the soil contains very high concentrations of these two nuclides and reported a significantly higher intake of these two elements by the population concerned.

2.2 SCE technique

Sister chromatid exchange is the reciprocal inter-change of DNA between chromatids and is easily visualised in metaphase chromosomes.

The first really decisive demonstration of the occurrence of equal symmetrical SCEs was made by Taylor (1958) in his autoradiographic studies of DNA replication and segregation in plant mitotic chromosomes.

Later studies showed that many of the SCEs observed in autoradiographic experiments were actually induced by the endogenous radiation from the incorporated tritium.

Zakharov and Egolina (1972) found that if they allowed cells to replicate for two rounds of DNA replication in the presence of bromodeoxy uridine (BrdU), then the chromosomes would contain one chromatid that would be unifiliarly substituted with BrdU and a sister chromatid that was

bifiliarly substituted. Such chromatids stained differentially with Giemsa so that exchanges between them could be readily seen.

Latt (1973) showed that if cells were grown in medium containing 5-BrdU for two cycles, the sister chromatids could be distinguished by the differential quenching of the fluorescence of the fluorochrome Hoeckst 33258.

Ikushima and Wolff (1974) found that other analogues of thymidine could cause the same effect.

Perry and Wolff (1974) devised a method to combine staining with a fluorescent dye plus Giemsa (FPG) or Harlequin chromosome method which allowed the detection of SCEs with far greater resolution and also the preparations were permanent.

Schneider *et al.* (1977) attempted to find out the number of previous cell replications that a metaphase cell had undergone in the presence of Brdu by the differential fluorescent patterns of metaphase chromosomes stained with Hoechst dye 33258. They also reported about the inhibitory effect of Brdu on cellular replication.

2.3 Agents inducing SCE

Sister chromatid exchange analysis is found to be the quickest, easiest and most sensitive mammalian system to test the genetic effects of mutagenic carcinogens.

Kato (1974) based on his autoradiographic studies, reported that alkylating agents could induce SCEs.

Latt (1974) found a four fold increase in SCE in BrdU treated human lymphocytes exposed to a chemical mutagen mitomycin C at a dose which caused only a low frequency (0.07/cell) of chromatid aberrations.

Perry and Evans (1975) studied the effect of twelve mutagens-carcinogens such as adriamycin, ethyl methane sulfonate, methyl methane sulfonate, mitomycin C, nitrogen mustard, 4-nitroquinoline 1-oxide, N-methyl-N-nitro-N-nitroso guanidine, diepoxy-butane, quinacrine mustard, cyclophosphamide, B-propriolactone and bleomycin and reported that all the above agents, except bleomycin, at a dose that gave a highly significant doubling of the incidence of SCE produced only a minimal and barely noticeable effect on the incidence of chromosomal aberrations. They found that doses giving ten fold rise in SCE gave increased, but still very low aberration frequencies.

According to Perry and Evans (1975), alkylating agents and in particular the bifunctional compounds-mitomycin C and nitrogen mustard were extremely potent inducers of SCE in CHO cells with mitomycin C giving a doubling of SCE frequency at 10^{-8}M. The monofunctional alkylating agents were generally less effective and exhibited a considerable range in their ability to induce SCEs.

Perry and Evans (1975) established a linear or curvilinear relationship between X-ray dose and SCE yield in G1 and S cells. But the increased incidence of SCE was minimal in relation to the induced incidence of chromosome and chromatid aberrations and this contrasted markedly with the results on chemical mutagens.

Perry and Evans (1975) found that the incidence of SCE in human blood lymphocytes was relatively constant between individuals and was independant of age and sex.

Soloman and Bobrow (1975) revealed that alkylating agents could induce SCEs at far lower concentrations though they did chromosome aberrations.

Stetka and Wolff (1976a) treated whole animals with test chemicals and then cultured their blood in the presence of BrdU to enable SCEs to be seen in the cultured lymphocytes.

When rat liver microsome activating system was incorporated with test chemicals in an *in vitro* test Stetka and Wolff (1976b) noticed that many of them were activated and could induce SCEs.

Vogel and Bauknecht (1976) and Allen and Latt (1976a) serially injected animals (mice) with BrdU and treated them with test chemicals (mitomycin C and cyclophosphamide). They counted the SCEs in bone marrow cells and observed significant induction of SCE with both test chemicals. Allen and Latt (1976b) followed the same procedure and observed increased frequency of SCEs in spermatogenia.

Pathak *et al.* (1977) found that the rate of SCE under identical conditions was the same in various mammalian species irrespective of their diploid numbers.

According to Takehisa and Wolff (1977) if CHO cells were treated with aflatoxin B1, the yield of SCE increased and in the presence of S-9 mix the increase was even more dramatic and occurred after a very short period of treatment with very low concentrations.

Wolff *et al.* (1977) noted that when human cells from patients with the disease *Xeroderma pigmentosum* in tissue culture were treated with low levels of mutagenic carcinogens, the yield of SCEs were greatly increased even at

concentrations of the chemicals that didn't increase the yield of SCEs in normal human cells.

Carrano *et al.* (1978) examined the relation between SCEs and mutations in CHO cells by quantifying the induction of SCEs in parallel with the induction of mutations producing 8-azaguanine resistance and their results indicated a positive linear relation between induced SCEs and mutations, but the relative efficiency of SCE and mutation induction varied with the chemical compound.

The yield of SCE per cell was dependant upon BrdU concentration in the media and also upon the concentration of target molecules i.e., cell density in the culture (Berardino and Shoffner, 1979).

Kram *et al.* (1979) described a new approach for detection of fetal DNA damage through the enumeration of SCEs in mouse fetal chromosomes.

According to Schvartzman *et al.* (1979), visible light illumination which had no apparent effect upon native DNA was able to increase the frequency of SCEs in 5-BrdU substituted chromosomes in *Allium cepa*.

Korte and Ruckert (1980) analysed the mycotoxins aflatoxin B_1 (AFB_1) and patulin (PA) with respect to their

chromosome breaking activity in Chinese hamster bone marrow *in vivo* and ranked in the order PA>AFB_1 with respect to the number of induced aberrant mitoses.

Allen *et al.* (1981) after conducting SCE analyses in maternal, embryonic and extra-embryonic tissues of pregnant rats and mice, proposed it as an assay to screen for fetal response to genotoxic chemicals.

Pereira *et al.* (1982) established that benzo [a] pyrene between 50 and 125 mg/kg administered maternally caused a dose-related increase in SCE in fetal hamster liver cells. There was no difference on days 11, 13 and 15 of gestation in the sensitivity of fetal liver to benzo [a] pyrene.

Curry *et al.* (1984) tested two naturally occurring fungal mycotoxins sterigmatocystin and griseofulvin for induction of SCEs in bone marrow cells of female Swiss albino mice. Sterigmatocystin gave elevated SCE frequencies at all doses tested (0.06-6.0 mg/kg). In contrast, griseofulvin tested from 0.4 to 200 mg/kg elevated SCE frequency only in those mice which received doses of 100 or 200 mg/kg body weight. These results indicated that both fungal mycotoxins induce SCE *in vivo* and are potentially mutagenic.

Natarajan and Mullenders (1987) proved that the induction of SCEs to be directly correlated with cytotoxicity.

Porter and Singh (1988) studied the transplacental fetotoxicity, teratogenicity and mutagenicity in Swiss Webster mice using different doses of cyclophosphamide and found that the increase in SCE per fetal liver cell was significant when compared to control.

Nair and Vogel (1989) conducted chromosome aberration test and SCE assay in murine bone marrow cells after *in vivo* exposure to D-penicillamine alone or combined with cyclophosphamide and confirmed the anticlastogenic effect of D-penicillamine.

Rupa *et al.* (1991) reported that the frequency of SCEs was significantly higher among a group of male pesticide applicators at all durations of exposure when compared to controls.

Wei *et al.* (1993) found that the increased frequency of SCE per cell concomitant with decreased frequency of 0-SCE cells was observed by raising the BrdU concentration in the culture medium during the first cell cycle, culturing cells at lower density and depleting reduced glutathione with buthionine sulfoximine.

Das *et al.* (1996) examined the applicability of using *Etroplus suratensis*, a brackish water fish species, as test

organism in screening genotoxic pollutants in aquatic environment and methods were evolved for demonstrating SCEs.

2.4 SCE in domestic animals

Bianchi *et al.* (1977) reported that the frequency of SCE in untreated pigs and rabbits were 7.27 ± 0.307 and 4.46 ± 0.158 per cell, respectively.

Berardino and Shoffner (1979) found out the frequency and location of SCEs in BrdU treated chromosomes from a population of healthy normal cattle of Holstein breed ranging from 18 to 24 months of age. They reported a mean SCE frequency of 5.4 ± 2.1 SCE per cell with a range of 1 to 16 per nucleus where the concentration of BrdU was 10 μg per ml in a cell density of $5x10^6$ lymphocytes per 1.5 ml of plasma. There was no difference in the frequency of SCE between males and females. According to them the yield of SCE in the late replicating X chromosome (facultative heterochromatin) was 2.5 times that of the early replicating homologue (active X).

Leibenguth and Thiel (1986) recorded SCE rates of 4.50 to 12.24 per metaphase and of 0.087 to 0.21 per chromosome after analysing the blood samples from nine German Black Pied and three German Red Pied cattle.

Iannuzzi *et al.* (1988) reported the average number of SCEs per cell as 8.8 q 3.4 for buffaloes.

Matseikene (1989) noted that the number of SCEs per metaphase cell averaged 5.81 and 4.92 in Lithuanian Red and Lithuanian Black Pied bulls, respectively.

For Murrah buffaloes, an SCE frequency of 3.66 per cell or 1.83 per cell per generation was recorded by Vijh *et al.* (1991a). There was no significant difference between the two sexes. The SCE frequency ranged between 2 and 7. He used a medium without antibiotics and antifungal agents and the level of incorporation of BrdU was limited to 5 μg/ml.

Vijh *et al.* (1991b) estimated the baseline SCE frequency in Black Bengal breed of goat by following a procedure where no antibiotics and antifungal agents were added to the culture medium. They reported a mean SCE frequency of 4.05 per cell or 2.025 per cell per generation and there was no significant difference between the sexes. It was also revealed that SCEs were non-random and smaller chromosomes exhibited relatively few exchanges.

Iannuzzi *et al.* (1991) reported that the incidence of SCE in Podolian, Friesian and Romagnola breeds of cattle which were reared under similar conditions were 7.9 q 3.4, 7.1 q 3.3

and 7.3 q 3.2, respectively, the difference between Podolians and Friesians being significant.

Vijh *et al.* (1992) estimated the baseline SCE frequency in Sahiwal cattle (*Bos indicus*) by following a protocol where no antibiotics and antifungal agents were added to the culture medium. They reported a baseline frequency of 3.17 per cell or 1.59 per cell per generation. There was no significant difference between the two sexes. The number of SCEs per metaphase cell ranged between 1 and 8 and the sites of distribution of SCEs were non-randomly distributed.

Sikka *et al.* (1993) noted that the average SCEs per cell cycle were 5.8 ± 0.28 and 5.3 ± 0.32 in Brucella positive and control animals, respectively. The range of SCEs per cell was similar in both groups (4 to 12 SCEs per cell) and there was no significant difference among the two groups of animals.

According to Catalan *et al.* (1994) the average number of spontaneous incidence of SCEs was 5.77 q 0.82 per cell in cattle.

Berardino *et al.* (1995) reported that in cattle (*Bos tarus*) the mean rate of spontaneous SCE per cell at 0.1 μg BrdU was 2.48 q 1.75 increasing to 5.16 q 2.55 at 5.0 μg BrdU after culturing perpheral blood lymphocytes.

Catalan *et al.* (1995) after analysing breed, sex and BrdU dose effects on SCEs in cattle reported that breed and BrdU dose factors had significant effects on SCE frequency and no differences between sexes were found. According to him, the breed of cattle as well as BrdU dose chosen for analysis must be considered when SCE test was used for the biomonitoring of environmental mutagens.

Vijh *et al.* (1995) noted a basal SCE frequency of 5.56 per cell in Bhadawari buffalaoes, the mean frequency for male and female buffaloes were 6.4 and 4.3 per cell, respectively.

Berardino *et al.* (1996) reported that the spontaneous level of SCE in goat, estimated by exposing peripheral blood lymphocytes to 0.1 μg per ml of BrdU was 3.28 ± 1.71 SCE per cell, 1.64 SCE per cell generation and 0.027 SCE per chromosome. The dose-response curve of SCE per cell observed by exposing the cells to 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 μg/ml of BrdU rose rapidly from 0.1 to 0.57 μg/ml, remained fairly stable from 0.5 to 1.0 μg/ml and rose less rapidly from 1.0 and 5.0 μg/ml of BrdU.

The baseline SCE frequency of Murrah buffaloes was 7.61/cell, ranging from 2 to 14 as reported by Joshi *et al.* (1996). The mean SCE frequency in males and females was 7.28 and 7.95 per cell, respectively.

Vijh *et al.* (1996a) reported that the over all mean SCE frequency of Rathi cattle as 4.06 per cell. The mean SCE frequency was 4.11 per cell for males and 4.00 per cell for female animals. The SCE varied from 1 to 8 per cell. The cultures in this study were set up without using antibiotics and anti-fungal agents to avoid any rise in the SCE frequency on this account.

Vijh *et al.* (1996b) reported a mean SCE frequency in Barbari goat as 3.78 per cell or 1.89 per cell per generation. There was no significant difference between the two sexes.

Materials and Methods

MATERIALS AND METHODS

In Kerala along the west coastal region, a stretch of land in Chavara Panchayat of Kollam district is well known as a source of monazite. In this area monazite is admixed with ilmenite, rutile, zircon and other rare earths. Here thorium and its decay products contribute to the high background radiation.

The four coastal wards of Chavara Panchayat, Cherusseribhagam, Kolangarabhagam, Karithura and Kovilthottam formed the area of study. A sample of 50 animals in the age group of 2 to 3 years were randomly selected so as to get a cross-section of the cattle population of this area. Ten animals, selected at random from the University Livestock Farm (ULF), Mannuthy where no radio-activity has so far been reported, formed the control group.

3.1 Sister Chromatid Exchanges (SCEs)

The procedure followed for SCE studies by Goswami (1982) was adopted with some modifications:

3.1.1 Reagents

3.1.1.1 Culture medium

Culturing of blood was done in a composite tissue culture medium having the following composition.

TC 199 or RPMI 1640 (Sigma)	= 1 g
Benzyl Penicillin solution (50,000 IU/ml)	= 0.2 ml
Phytohemagglutinin-M solution (2000 μg/ml - Sigma)	= 0.5 ml
Pokeweed mitogen solution (1000 μg/ml - Sigma)	= 1.0 ml
Sodium bicarbonate solution (3.5%)	= 1.0 ml
Double distilled water (autoclaved)	= 97.3 ml

The pH of the medium was adjusted to 6.8 to 7.2 with sodium bicarbonate solution (3.5%) or N/10 hydrochloric acid. The medium was filtered through membrane filter (pore size = 0.22 μ) and then it was divided into 5 ml aliquots in culture vials and stored at -5°C.

Cultures were prepared using both TC 199 and RPMI-1640 and efficiency of the media was evaluated based on mitotic index and mitotic drive.

3.1.1.2 5-Bromo-2-deoxy uridine (BrdU) solution

Stock solution

Fourteen milligram (mg) of BrdU was dissolved in 10 ml tissue culture medium.

Working solution

One millilitre stock solution was mixed with 9 ml tissue culture medium to get a final concentration of 70 μg per 0.5 ml solution.

Both the vials containing stock and working solutions were wrapped in aluminium foil to prevent exposure to visible light.

3.1.1.3 Thionine solutions

Thionine (23 mg) was dissolved in 100 ml double distilled water, filtered and stored in amber coloured bottle.

3.1.1.4 Sodium phosphate (0.16 M) - Soidum citrate (0.05 M) buffer

(i) Sodium phosphate dibasic (227.2 mg) was dissolved in double glass distilled water and volume made to 10 ml.

(ii) Sodium citrate (117.64 mg) was dissolved in double distilled water and volume made to 10 ml.

Solutions (i) and (ii) were mixed together and pH adjusted to 7.0

3.1.1.5 Heparin sodium

One millilitre of heparin sodium (1000 IU/ml) was diluted with one millilitre of sterile distilled water. Using a 20 G needle and syringe, two drops of anticoagulant was added in each collection vial.

3.1.2 Collection and transportation of blood

Ten millilitres of blood was collected from the jugular vein of animals using sterile 16 G needles under aseptic conditions. Heparin sodium was used as anticoagulant.

Blood samples were transported from the field to the laboratory in a thermoflask packed with ice. Care was taken to avoid direct contact between ice and collection vials.

3.1.3 Culturing for SCE studies

Method 1: Whole blood (0.5 ml) was added to each culture bottle containing 5 ml tissue culture medium, prewarmed at 37°C. Two millilitres of autologous plasma, obtained by centrifuging the samples at 1200 rpm for 15 minutes was added to each culture. After gentle mixing, the culture bottles were incubated at 37°C for 20 hours. During incubation the vials were mixed twice daily to avoid sedimentation. At the end of 20th hour of incubation 0.5 ml of BrdU working solution was added to each tube. The incubation was continued at 37°C for another 50 hours in dark.

At the end of 70th hour of incubation the cultures were harvested.

Method 2: Whole blood (0.5 ml) was added to each culture bottle containing 5 ml tissue culture medium, prewarmed at 37°C. Two millilitres of autologous plasma, obtained by

centrifuging the samples at 1200 rpm for 15 minutes was added to each culture. Then 0.5 ml BrdU working solution was added to each tube (i.e. at the commencement of culturing itself). After gentle mixing, the culture bottles were incubated in dark for 48 hours at 37°C. During incubation the vials were mixed gently twice daily to avoid sedimentation.

At the end of 48th hour, the cultures were harvested.

3.1.4 Harvesting

The following procedure was undertaken to prepare metaphase spreads on glass slides.

(i) One drop of colchicine (sigma) solution (10 μg/ml) was added using a 20 G needle and syringe to each culture and incubated for one hour at 37°C.

(ii) The cultures were centrifuged at 1200 rpm for 15 minutes.

(iii) The supernatant was discarded and the cell button was resuspended in 0.56 per cent potassium chloride (0.075 M) prewarmed to 37°C to make 6 ml of cell suspension.

(iv) The suspension was incubated for 10 minutes at 37°C.

(v) The suspension was centrifuged at 1200 rpm for 15 minutes.

(v) The supernatant was discarded and the cell button was resuspended with 5 ml of chilled methyl alcohol-acetic acid fixative (3:1) and left undisturbed for 15 minutes.

(vii) The suspension was centrifuged at 1200 rpm for 15 min.

(viii) The supernatant was discarded and the cell button was resuspended in 5 ml of methyl alcohol-acetic acid fixative.

(ix) Step (vii) and (viii) were repeated till the supernatant was clear.

(x) The supernatant was discarded and a cell suspension of about one millilitre was made using fresh fixative.

During harvesting care was taken to limit exposure of cells to visible light.

3.1.5 Preparation of slides

Clean grease-free slides chilled in cold methanol were used. Four drops of cell suspension were dropped on to each slide while keeping them in a slanting position from a height of about half to one metre. The spreads were fixed on flame and the slides were dried and kept in a box for 3 to 5 days for aging.

3.1.6 Sister chromatid differential staining

3.1.6.1 Method suggested by Goswami (1982) was used for sister chromatid differential staining.

(i) Three to five days old slides were dipped for 15 to 30 seconds in single distilled water.

(ii) These slides were stained with thionine for 10 minutes, washed in distilled water and air dried.

(iii) Three drops of sodium phosphate (0.16 M) - Sodium citrate (0.05 M) buffer was placed on the slides and covered with coverslips and sealed with molten wax.

(iv) The slides were exposed to sunlight for two hours.

(v) After removing the coverslips, slides were rinsed with single glass distilled water.

(vi) The slides were stained with 2.0 per cent Giemsa stain for 10 minutes, rinsed with distilled water, air dried and kept for incubation at 37°C for 24 hours. The slides were screened for SCEs.

3.1.6.2

(i) Three to five days old slides were dipped in single glass distilled water for 30 seconds.

(ii) These slides were stained with thionine for 40 minutes, then washed in distilled water and air dried.

(iii) The slides were exposed to ultra-violet (UV) light after flooding them in single glass distilled water for 60 minutes. Trials were made with one, one and half and two feet distance from UV source.

(iv) The slides were incubated at 37°C for 24 hours.

(v) The slides were stained with Giemsa (10%) for 12 min, rainsed with distilled water and air dried. The slides were screened for SCEs.

3.1.7 Microscopy and photography

At least three slides from each culture were screened under microscope for metaphase spread showing differential staining. About 10 spreads of each animal were studied for SCEs.

Good spreads showing SCEs were photographed using Carl-Zeiss photomicroscope III. The photographs were taken on 100 ASA black and white film.

Results

RESULTS

The role of chemicals and radiation in causing genetic damage is well recognised. An exploration on chromosomal damage in cattle exposed to high background radiation would help to assess the effect of long-term radiation on the performance and behaviour pattern of animals.

The Chavara belt which is well known for its background radiation was the area of study. This area has very high deposits of radioactive Thorium^{232} rich monazite sand. The quantification of radioactivity revealed that there exists a background radiation which is more than 250 times that of the normal background radiation levels of 0.02 m R/hr. This area supports a large cattle population which includes both cross-breds and non-descript animals (Table 1).

The reports revealed that the intensity of radiation decreases from the shoreline to in-land areas. Hence cattle reared in the four coastal wards of Chavara Panchayat viz. Cherusseribhagam, Kolangarabghagam, Karithura and Kovilthottam were studied and compared with a control group of cattle reared in the ULF, Mannuthy.

Sister chromatid delineation and its evaluation is considered as an indicator of chromosomal damage. The

quantification of SCE involves standardization of technique which distinguishes each SCE.

The blood samples were collected from jugular vein and subjected to lymphocyte culture. Phytohemagglutinin-M (2000 μg/ml) and Pokeweed mitogen (100 μg/ml) were used as mitogens. The efficiency of TC-199 and RPMI-1640 as tissue culture media were studied using mitotic index and mitotic drive (Table 2).

The chromosomes were tagged with thymidine base analogue 5-bromodeoxy uridine (5 BrdU) and analysed for SCE.

In the first method, 0.5 ml BrdU working solution (140 μg/ml) was added at 20th hr of incubation and cultures were harvested at the end of 70th hr after one hour colchicinisation.

In the second method, 0.5 ml BrdU working solution (140 μg/ml) was incorporated at commencement of culturing and harvesting was carried out at the end of 48 hrs after one hour colchicinisation.

The incorporation of Brdu at 20th hr yielded better cell proliferation.

The harvested cells were used for the preparation of metaphase spreads on glass slides. Three to five days old

slides were subjected to differential staining for SCE analysis as follows.

(a) The air dried slides were stained with thionine for 10 min and dried. A few drops of phosphate - citrate buffer was placed on the slide and covered with a coverslip and edges were sealed with molten wax. These slides were exposed to sunlight for 2 hrs. Then the coverslips were removed and dried slides were stained with Giemsa (2%) for 10 min. The slides were rinsed with distilled water, dried and kept in incubator at 37°C for 24 hrs. These slides were examined for SCE.

(b) The air dried slides were stained with thionine for 40 min, washed and dried. Then the slides were exposed to UV-light (the slides were kept at a distance of one feet from the UV source) after flooding them in single glass distilled water for 60 min. The slides were incubated at 37°C for 24 hrs and then stained with Giemsa (10%) for 12 min. These slides were examined for SCE.

The second method of sister chromatid differential staining yielded distinct SCEs.

Thus the technique standardized for SCE visualisation in this laboratory involves culturing of lymphocytes in RPMI-1640 medium and incorporation of BrdU at 20th hr of incubation.

Harvesting of cells were done at the end of 70th hr and then exposing the thionine stained slides to UV light for one hour at a distance of one feet from the UV source.

The chromosome spreads were prepared for cattle of both control and experimental groups. Those metaphase spreads showing sister chromatid differential staining were analysed for SCE frequency. A photograph of metaphase spread showing SCEs is presented in Plate 1. Chromosomes with Sister chromatid differentiation (SCD) and SCE are diagramatically represented in Fig.1.

The number of spreads with SCD and SCE were scored for experimental and control areas and presented in Table 3. The frequency of cells with different levels of SCEs were analysed (Fig.2).

The mean SCE frequency/cell was 1.536 ± 0.249 for control group and 3.368 ± 0.273 for cattle from radiation affected area. The SCE frequency was varied from 0 to 5 and 0 to 9 for control and experimental group, respectively. For control, the SCE/cell/generation was 0.768 and for experimental animals, it was 1.684.

The results of analysis for SCE frequency for cattle from the four coastal wards of Chavara Panchayat (Kovilthottam,

Cherusseribhagam, Kolangarabhagam and Karithura) and control (ULF, Mannuthy) were given in Table 4.

a. **Kovilthottam**

Kovilthottam lies in the northern part of Chavara Panchayat. Cattle of this area appeared healthy. The mean SCE frequency per cell was found to be 4 ± 0.966 with a range of 0 to 7. The exchanges per cell per generation was 2.0.

b. **Cherusseribhagam**

It lies south of Kovilthottam. Cattle were phenotypically healthy. The mean SCE frequency was 2.563 ± 0.584 and the number of SCEs per cell varied from 0 to 6. The frequency of exchanges/cell/generation was 1.282.

c. **Kolangarabhagam**

This ward lies between Cherusseribhagam (North) and Karithura (South). A mean SCE frequency of 3.206 ± 0.411 was obtained for cattle from this ward. The number of exchanges per cell varied form 0 to 7 and exchanges per cell per generation was 1.603.

d. **Karithura**

Karithura ward lies in the southern part of Chavara Panchayat. The animals were appeared healthy. The exchanges

per cell varied from 0 to 9 and the mean frequency was 3.839 ± 0.504. The number of exchanges per cell per generation was 1.920.

e. Control (ULF, Mannuthy)

Cattle from University Livestock Farm, Mannuthy were taken as control. The mean SCE frequency was 1.536 ± 0.249 and range was 0 to 5. The frequency of exchanges per cell per generation was 0.768.

Statistical analysis

The data obtained were subjected to analysis using standard statistical techniques (Das and Giri, 1979). The SCE frequencies of cattle in control and radioactive coastal area were compared. The SCE frequencies of cattle in radioactive belt area of Chavara Panchayat exhibited a significant increase when compared to that of control control animals. It was also revealed that the four wards of Chavara Panchayat (Kovilthottam, Cherusseribhagam, Kolangarabhagam and Karithura) did not exhibit a significant difference in SCE frequency among themselves. However, difference in SCE frequency between Cherusseribhagam and control was not significant.

Table 1. Cattle population in Chavara Panchayat (as on February, 1996)

	Female				Males	Total
	Less than 1 yr	1 to 2½ hr	Above 2½ yr	Total		
Crossbred	367	272	803	1442	50	1492
Non-descript	70	75	169	314	11	325
Total	437	347	972	1756	61	1817

Table 2. Mitotic index and mitotic drive observed in peripheral blood lymphocytes of cattle

Types of culture	Mitotic activity				
	Lympho-blasts	Mitotic spreads	Total cells	Mitotic index (%)	Mitotic drive (%)
TC-199 medium	44	4	160	2.5	30
RPMI-1640 medium	77	11	210	5.24	42

Table 3. Frequency of cells showing SCEs in cattle in control and radioactive areas

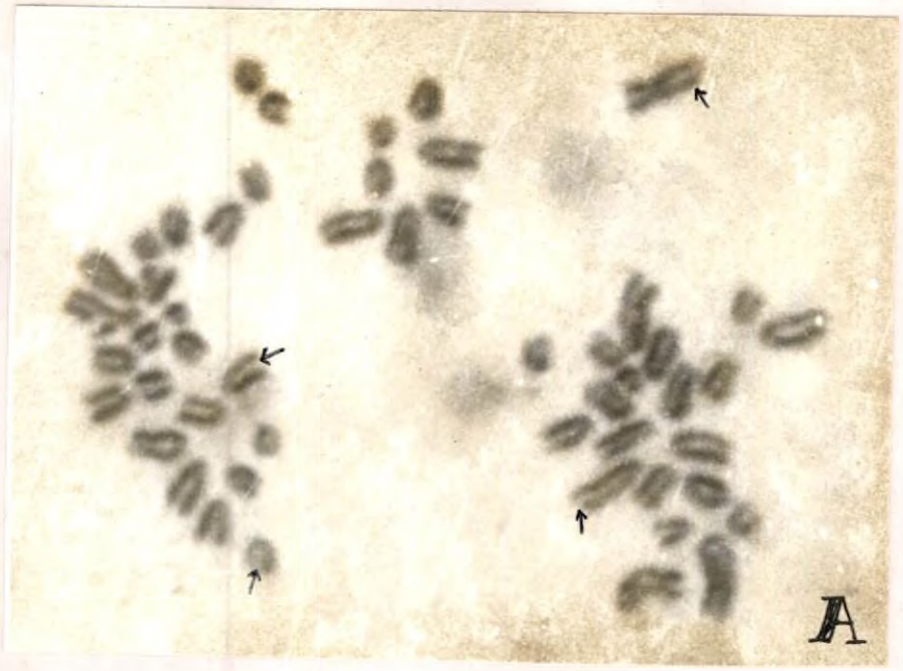
No. of SCEs per cell	Frequency of cells showing different No. of SCEs				
	Karithura	Kolangara-bhagam	Kovil-thottam	Cherusseri bhagam	Control
0	6	9	1	3	8
1	2	-	-	5	5
2	4	4	-	1	10
3	2	5	1	2	3
4	3	3	1	1	1
5	5	7	2	-	1
6	4	3	-	4	-
7	1	3	1	-	-
8	2	-	-	-	-
9	2	-	-	-	-
Total no.of cells	31	34	6	16	28

Table 4. Range and mean SCE frequencies of cattle reared in radioactive (A) and control (B) areas

Area	No. of animals	No. of spreads with SCD	SCE/cell		SCE/cell/ generation
			Mean	Range	
(A) Karithura	12	31	3.389± 0.504	0-9	1.920
Koulangara-bhagam	17	34	3.206± 0.411	0-7	1.603
Kovilthottam	9	6	4.000± 0.966	0-7	2.000
Cherusseri-bhagam	12	16	2.563± 0.584	0-6	1.282
	50	87	3.368± 0.273	0-9	1.684
(B) Control	10	28	1.536± 0.249	0-5	0.768

Plate 1. **Sister chromatid exchanges of cattle reared in radio active belt area of southern Kerala**

A B


A


B

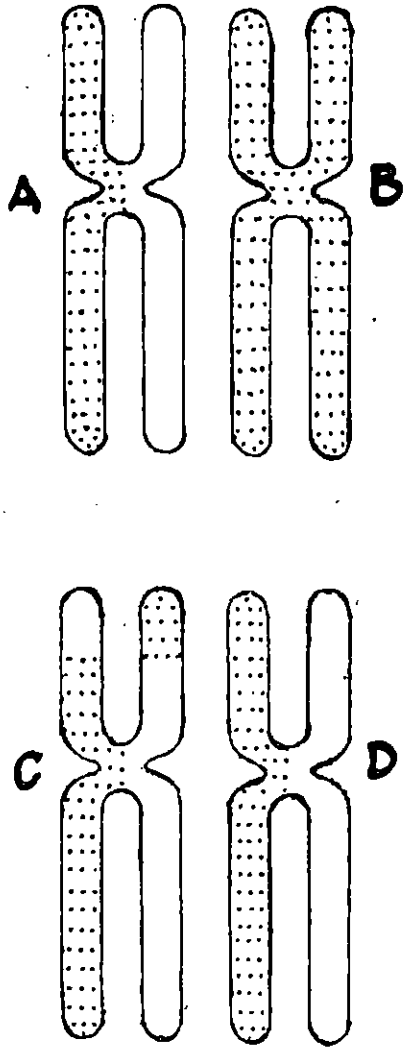
Plate 1. **Sister chromatid exchanges of cattle reared in radio active belt area of southern Kerala**

C D


C

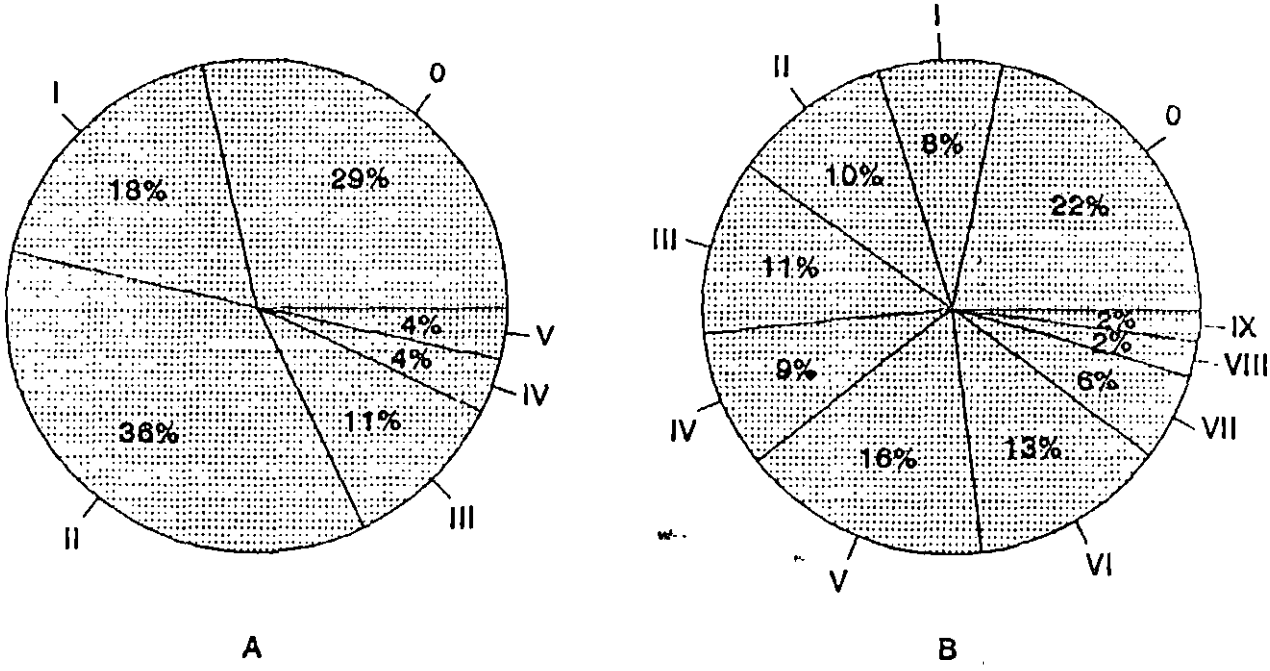

D

Fig.1. **Diagramatic representation of sister chromatid exchanges in Cattle**



A- Chromosome with SCD
B- Chromosome without SCD
C- Chromosome with SCE
D- Chromosome without SCE

Fig.a DISTRIBUTION OF CELLS WITH DIFFERENT SCE FREQUENCY IN CONTROL AND RADIOACTIVE AREAS



A- Control,
B- Radio active area,
0 to IX- No. of SCE/cell

Discussion

DISCUSSION

A cytogenetic investigation on the effect of long-term background radiation on cattle was carried out in this study. The investigation involved analysis of SCEs which was considered as the most sensitive and quickest mammalian system to find out the effect of mutagens on genetic material.

5.1 Background radiation

Areas with naturally occurring high background radioactivity exists along the coastal stretches of Southern Kerala. This area has very high deposits of radioactive Thorium^{232} rich monazite sand. Various reports revealed that along this coastal belt the background radiation reached its maximum level in Chavara area. Ayengar *et al.* (1971) reported that fraction of households receiving greater than 5-10 times normal background radiation exposure levels was highest in Chavara area.

The role of thorium^{232} and its decay products as a source radiation was reported by Ayengar *et al.* (1970). During the decay of thorium^{232} alpha, beta and gamma radiations are released and also radioactive thoron which diffuses out of the sands and contributes to the contamination of air. Therefore

cattle population living in this area is subjected to radiation exposures from (a) external radiations by beta and gamma rays from natural uranium and thorium contained in monazite, (b) beta and gamma radiations from radon, thoron and their decay products in the air and (c) internal exposures from deposition of these radioactive materials in the body through ingestion and inhalation.

In the present study cattle from the four coastal wards of Chavara Panchayat (Kovilthottam, Cherusseribhagam, Kolangarabhagam and Karithura) were taken as the experimental group. Cattle from ULF, Mannuthy which has no reports of background radiation was considered as the control group.

5.2 SCE technique

Sister chromatid exchange technique was used to detect the effect of high background radiation on chromosomes. For SCE analysis blood lymphocytes were cultured in RPMI-1640 tissue culture medium containing mitogens (Pokeweed and Phytohaemagglutinin-M), antibiotics (Penicillin) and a thymidine analogue BrdU. When the cells were allowed to replicate for two generations in the presence of BrdU, the chromosomes contained one chromatid which was unifiliarly substituted. When metaphase spreads were prepared and stained

by thionine followed by Giemsa and exposed to UV light, the sister chromatids were differentially stained and exchanges could be visualized. The technique of thionine-plus-Giemsa staining for SCD was explained by Goto *et al.* (1975), Sikka *et al.* (1993) and Sahai and Vijh (1995). The use of fluorescent dye Hoechst 33258 and Giemsa in fluorescent dye-plus-Giemsa (FPG) staining was reported by many scientists (Perry and Wolff, 1974, Zakharov and Egolina, 1972, Latt, 1973, Allen and Latt, 1976, Perry and Evans, 1975, Das *et al.*, 1996, Vijh *et al.*, 1991, Vijh *et al.*, 1992 and Vijh *et al.*, 1996).

The effect of BrdU incorporation at different times of cell culturing was studied. In the first, 0.5 ml BrdU working solution (140 μg/ml) was added at 20th hr and incubated for another 50 hrs. In the second, same amount of BrdU working solution was added at the commencement of culturing and incubated for 48 hrs. Later the cells were harvested for SCE analysis. It was found that addition of BrdU at 20th hr yielded more lymphoblasts and metaphase spreads. Goswami (1982) also reported addition of BrdU at 20th hr of incubation to get better SCEs. Addition of BrdU at the commencement of culturing resulted poor yield of lymphoblasts and this might be due to the inhibitory effect of BrdU on initiation of cell replication. Schneider *et al.* (1977) reported the inhibitory effect of BrdU on cell replication.

In the present study, the exposure of thionine stained slides to UV light for one hour at a distance of one feet from the source yielded good SCEs. Sahai and Vijh(1995) exposed the slides to sunlight for 2 hrs to yield SCEs.

5.3 SCE frequency

The number of SCEs per cell was determined. The mean SCE frequency was found to be 1.536 ± 0.249/cell for control group. The SCE range was 0 to 5 and number of SCEs/cell/ generation was 0.768.

This baseline frequency obtained was less than that reported by many scientists. Berardino and Shoffner (1979) got a higher baseline value of 5.4 ± 2.1 SCE/cell in cattle. This might be due to a higher level of BrdU used. Catalan *et al.* (1995) reported that BrdU dose levels had significant effects on SCE frequency. Berardino *et al.* (1995) reported that SCE frequency was increased as the concentration of BrdU enhanced.

Vijh *et al.* (1992) and Vijh *et al.* (1996) reported higher values of SCE baseline frequencies in cattle. In their study cells were allowed to replicate for one more generation after BrdU incorporation than in the present study. In 1984, Natarajan and Mullenders established that BrdU labelled DNA

was highly prone to SCEs and explained that there exists a positive correlation between the frequency of SCEs and amount of BrdU incorporated in the DNA.

The difference in SCE frequency for various breeds of cattle was reported by many scientists (Matseikene, 1989, Leibenguth and Thiel, 1986, Iannuzzi *et al.*, 1991 and Catalan *et al.*, 1994). Catalan *et al.* (1995) revealed that breed and BrdU dose levels play significant roles on SCE frequency.

In the present study, mean SCE frequency for cattle reared in areas with background radiation was 3.368 ± 0.273 where as it was only 1.536 ± 0.249 for control group. Thus an increase of 119.27 per cent in SCE frequency was recorded in radiation affected area. The range of SCE varied from 0 to 9 in the experimental group whereas it was from 0 to 5 in control animals. This could be attributed to the effect of radioactive rays on increasing SCE frequencies by damaging DNA and chromosomes. Perry and Evans (1975) reported that X-ray induced lesions could manifest as SCE events as a consequence of DNA replication. However, Natarajan and Mullenders (1984) reported that though ionizing radiations were potent inducers of chromosome aberrations and DNA strand breaks were poor inducers of SCEs.

The effect of many chemicals and carcinogens were evaluated and explained on induction of SCEs (Kato, 1974,

Latt, 1974, Perry and Evans, 1975, Soloman and Bobrow, 1975, Takehisa and Wolff, 1977, Wolff *et al.*, 1977, Allen *et al.*, 1981, Pereira *et al.*, 1982, Curry *et al.*, 1984, Porter and Singh, 1988, Nair and Vogel, 1989, Rupa *et al.*, 1991, Wei *et al.*, 1993 and Das *et al.*, 1996). Perry and Evans (1975) reported that the marked increase in the incidence of SCE in cells exposed to a wide variety of chemical mutagens was associated with very much smaller increases in chromosome aberration yields whereas the converse holds in cells exposed to X-radiations.

5.4 SCE frequencies in individual wards

The four wards of Chavara Panchayat were analysed. The mean SCE frequencies for cattle in Kovilthottam, Cherusseribhagam, Kolangarabhagam and Karithura were 4 ± 0.966, 2.563 ± 0.584, 3.206 ± 0.411 and 3.839 ± 0.504, respectively. The SCE range recorded in the above wards were 0 to 7, 0 to 6, 0 to 7 and 0 to 9 respectively. The difference between SCE frequencies of Kovilthottam, Kolangarabhagam and Karithura to that of control was significant though among the four wards there was no significant difference. However, the difference in SCE frequency between Cherusseribhagam and control was not significant. Ayengar *et al.* (1971) reported that along the

coast the high radiation belt was frequently interrupted by stretches with normal background radiation levels and this large variation was chiefly due to non-homogenous distribution of monazite deposits in the coastal belt. This might be the reason for insignificant difference between SCE frequencies of control and that of Cherusseribhagam.

In this study it was found that the percentage increase in SCE for cattle in Karithura, Kolangarabhagam and Kovilthottam over that of control were 149.94 per cent, 108.72 per cent and 160.42 per cent. This increase though statistically significant was not indicative of high background radiation to which the animals were exposed.

The present study using SCE frequency analysis as indicative of chromosome damage revealed that there exists a significant increase in SCE frequencies in cattle reared in areas with long-term high background radiation in comparison to control areas. Thus it indicates that there is a disturbance or damage in genetic material at chromosomal or DNA level though the effect could not be established as very high. Further, observations on performance aspects of cattle in these areas did not exhibit any deviation in physiological or health status from normal population. This may be due to chromosomal damage or mutation at low frequencies are being

excellently repaired naturally or balanced by exchange mechanism during active replication of chromosomes. Thus the inference shows that there is chromosomal lesions produced by continuous background radiation though their expression is not seen as physiological or chromosomal abnormalities. The accumulation of such lesions on genetic material due to long-term radiation exposure warranty the genetic risk involved in it.

Summary

SUMMARY

1. Information on the effect of long-term high background radiation on genetic material was analysed in the present study.

2. A stretch of coastal land of Chavara Panchayat of Kollam district with sand containing monazite admixed with ilmenite, rutile, zircon and other rare earths formed the site for study. Here thorium^{232} and its decay products contribute to the high background radiation.

3. The phenomena of SCE involves exchange of parts between sister chromatids and it is considered as the simplest and most sensitive mammalian system to detect chromosomal damage.

4. A sample of 50 cattle in the age group of 2 to 3 years reared in four coastal wards of Chavara Panchayat viz. Kovilthottam, Cherusseribhagam, Kolangarabhagam and Karithura were subjected to chromosomal delineation and SCE analysis. Samples from ULF, Mannuthy without history of any background radiation formed the control group.

5. The technique for SCE analysis using blood samples from cattle was standardized.

6. Incorporation of 0.5 ml thymidine analogue, BrdU working solution (140 μg/ml) to culture at 20th hr of incubation yielded cell proliferation.

7. Differential staining using thionine and UV exposure for 60 minutes followed by staining with Giemsa (10%) yielded better SCD.

8. SCE frequency/cell, SCE frequency/cell/generation and range were recorded for cattle in both control and experimental groups.

9. For control animals, the SCE frequency/cell was 1.536 ± 0.249 and 3.368 ± 0.273 for experimental group whereas range of frequency was 0 to 5 and 0 to 9, respectively. The number of SCE/cell/generation was 0.768 for control and 1.684 for experimental animals.

10. Analysis of data revealed that there was a significant increase in SCE frequency in cattle of high background radiation belt when compared to that of control area.

11. SCE frequency/cell was 3.389 ± 0.504, 3.206 ± 0.411, 4.0 ± 0.966 and 2.563 ± 0.584 in Karithura, Kolangarabhagam, Kovilthottam and Cherusseribhagam wards of Chavara Panchayat, respectively.

12. The study revealed that difference in SCE frequency/cell between wards was not significant. The difference between SCE frequencies of Kovilthottam, Kolangarabhagam and Karithura to that of control was significant. However, there was no significant difference in the SCE frequencies of control and Cherusserybhagam.

13. Analysis of reports revealed that cattle of Chavara Panchayat exhibited normal physiological status.

14. This study using SCE analysis pointed out that background radiation caused damage or breaks on chromosome but these lesions are being repaired naturally at low frequencies.

15. Though these mutations or damages on genetic material are balanced at low frequencies,, long term background radiation warranty against genetic risk.

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EVALUATION OF SISTER CHROMATID EXCHANGES IN CATTLE REARED IN A RADIO ACTIVE BELT AREA OF SOUTHERN KERALA

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ABSTRACT OF A THESIS

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ABSTRACT

A cytogenetic investigation on the effect of long-term background radiation on cattle was carried out in this study. It involved the analysis of sister chromatid exchanges (SCEs) which was the most sensitive and quickest mammalian system to find out the effect of mutagens on genetic material.

The technique standardized for sister chromatid differentiation (SCD) involved culturing of lymphocytes in RPMI-1640 medium and incorporation of 5-bromo-2-deoxy uridine (BrdU) at 20th hour of incubation. The cells were harvested at the end of 70th hour and the thionine stained slides were exposed to UV light for one hour at a distance of one foot from the UV source. Metaphase spreads showing SCD were analysed for SCE.

Cattle reared in the four coastal wards of Chavara Panchayat (Kovilthottam, Cherusseribhagam, Kolangarabhagam and Karithura) were taken as experimental group. Cattle from University Livestock Farm (ULF), Mannuthy, which has no reports of background radiation, formed the control group.

The mean SCE frequency per cell was found to be 1.536 ± 0.249 and 3.368 ± 0.273 for control and experimental groups,

respectively. The range and number of SCEs/cell/generation for control animals were 0 to 5 and 0.768, respectively. In the experimental group it was 0 to 9 and 1.684. Thus an increase of 119.27 per cent in SCE frequency was recorded in high background radiation area when compared to that of control.

The mean SCE frequencies for cattle of Kovilthottam, Cherusseribhagam, Kolangarabhagam and Karithura were 4 ± 0.966, 2.563 ± 0.584, 3.206 ± .411 and 3.389 ± 0.504, respectively and the SCE ranges were 0 to 7, 0 to 6, 0 to 7 and 0 to 9. There was no significant difference in SCE frequency among the four wards. The difference between SCE frequencies of Kovilthottam, Kolangarabhagam and Karithura to that of control was significant. This could be due to the effect of high background radiation on DNA and chromosomes. However, difference in SCE frequency between Cherusseribhagam and control was not significant. This could be due to non-homogenous distribution of monazite deposits in the coastal belt.

Though the increase in SCE frequencies in Karithura, Kolangarabhagam and Kovilthottam were statistically significant, cattle reared in this area did not reveal any deviation in physiological and phenotypic performance.

Thus this study indicates that SCE frequency for cattle reared in Chavara panchayat with high background radiation

was significantly higher than that of control group. This discloses the occurrence of chromosomal damage in this area though these cattle performed normally. This may be because of the repair mechanism or balanced by exchange mechanism during active replication of chromosomes.