Study of *in vitro* Genotoxicity of Nanoparticles: Cellular uptake, DNA interaction, and chromosome breakage

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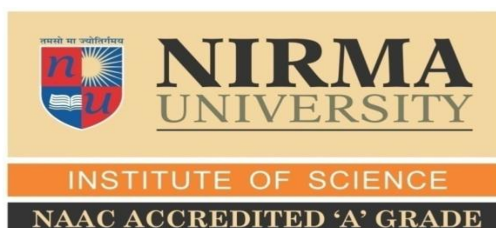

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INTRODUCTION

1.1 NANOPARTICLES

The progress in nanotechnology has led to tremendous increase in application of nano materials. In a range of consumer products including colloidal health drinks, sunscreens, cosmetics, carbon fiber sports equipment, electronic products, and as antibacterial components of toys, wound dressings and food additives.

This has increased the human exposure to these Nano materials through inhalation, ingestion, and dermal penetration [1]. Nanoparticles have unique physicochemical properties due to their small size, large surface area and high reactivity [2]. Nano sized Particles are more biologically active than their micron sized counterparts. The toxicity of nanoparticles (NPs) is influenced by physicochemical characteristics such as: the primary particle size, agglomeration state, specific surface area, zeta potential, and surface chemistry [8]. The small volume and large relative surface area of nanoparticle scan give rise to a number of properties that deviate dramatically from those found in their bulky counterparts. [6] In October 2011 the European Union defined Nano materials as a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or agglomerate; where 50% or more of the particles exhibited, one or more external dimensions in the size range 1–100 nm. Some have defined nanoparticles (NPs) as, at least one of the three dimensions in the range of 1–100 nm. The larger surface to volume ratio imparts unique properties to NPs as compared to the bulk form of chemical making them highly preferred for various applications [1].

1.2 TITANIUM DIOXIDE (TiO_2) NANOPARTICLES

Titanium dioxide has three natural forms: rutile, Anatase, and brookite. TiO_2 NPs are widely used in cosmetics and sunscreen products which account for 50% of usage. They are also used as a photocell coating and as a photo catalyst to reduce waterborne or airborne pollutants such as organic dyes, formaldehyde, nitric oxide, benzene and organophosphorus insecticides because they are n-type semiconductor. They have been used in self-cleaning sanitary ceramics, cement, and antimicrobial plastic packaging. They are also used in window glass, pavement, and walls because light-mediated TiO_2 surface hydroxylation makes it fouling-resistant. They have also been used as additives in film, capsules, tooth-paste, and sugar.[9] According to the US National Nanotechnology Initiative TiO_2 nanoparticles are amongst the most manufactured, and are widely used in a broad range of products such as food colorants.[3] According to a report from the International Agency for Research on Cancer (IARC), TiO_2 nanoparticles are possibly carcinogenic to humans (Group 2B), which is based on sufficient evidence in experimental animals and inadequate evidence from epidemiology studies [13].

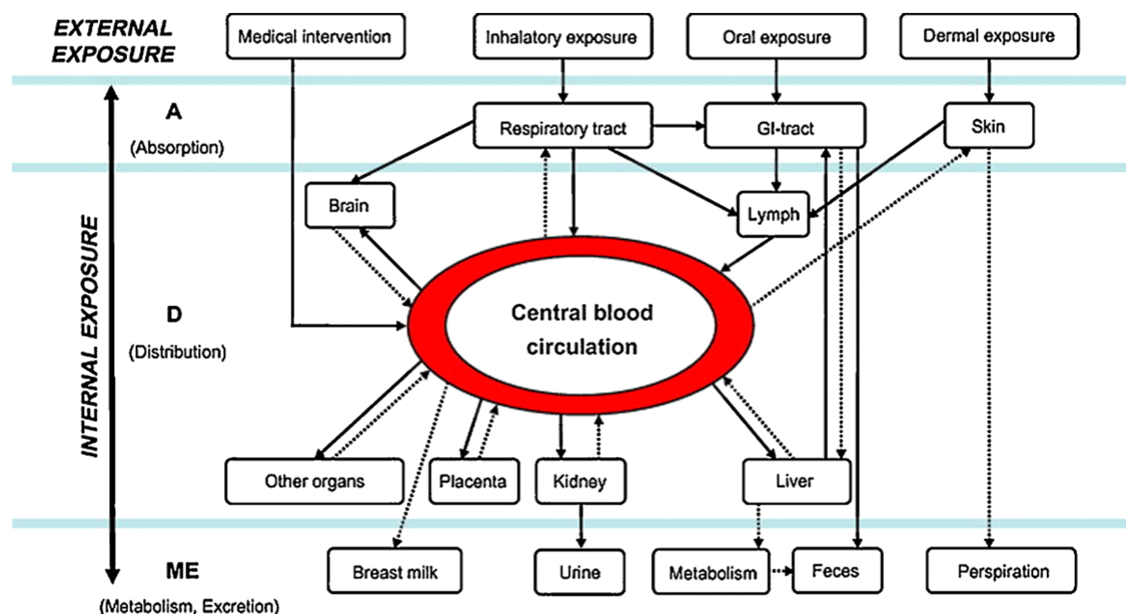


Fig.1: Kinetics of exposure of NPs in the body; internal exposure results from the portion of the external dose that enters the systemic circulation [Zhang, R. et al., 2011.]

1.3 TOXICITY STUDIES OF TiO_2 NPs IN VARIOUS ROUTES OF EXOSURE

- **Respiratory System**

As the exogenous fine particles enter inside the body, which could be mainly via the respiratory system, after which phagocytosis by alveolar macrophages induces reactive oxygen species (ROS). Alveolar macrophages usually contain enzymatic and non-enzymatic antioxidants that scavenge ROS; however, these are insufficient to prevent oxidative stress and pulmonary damages. TiO_2 NPs have been found to cause pulmonary damage and Inflammation. Acute exposure to TiO_2 NPs rods and dots for 24 h caused pulmonary and cardiac edema, lung and systemic inflammation, and platelet aggregation [9].

- **Skin**

TiO_2 NPs are widely used in sunscreens, cosmetics, and even clothes to provide protection from harmful UV irradiation. These applications cause general skin exposure, posing the potential risk of percutaneous absorption and ROS- mediated skin aging. Skin and hair follicle facilitates transdermal drug delivery but also provides a possible route for NP entry and a long-term NP reservoir. The stratum corneum (the upper layer of epidermis) shields against percutaneous penetration by most extraneous substances, but TiO_2 NPs were shown to penetrate the stratum corneum and the stratum

granulosum in pigs, however there is no evidence that NP skin penetration leads to systemic exposure [9].

- **Brain and CNS**

Because of low capacity for cellular regeneration, high metabolic rate, and numerous cellular ROS targets, brain is highly vulnerable to oxidative stress. The olfactory nerve is speculated to be the most likely pathway for the transport of intranasally instilled NPs to the brain. High accumulation, oxidative stress, and obvious morphological alteration of hippocampal neurons and olfactory bulb were detected after nasal exposure to TiO_2 NPs. In contrast, TiO_2 NPs induced only a slight brain lesion after oral gavage [9].

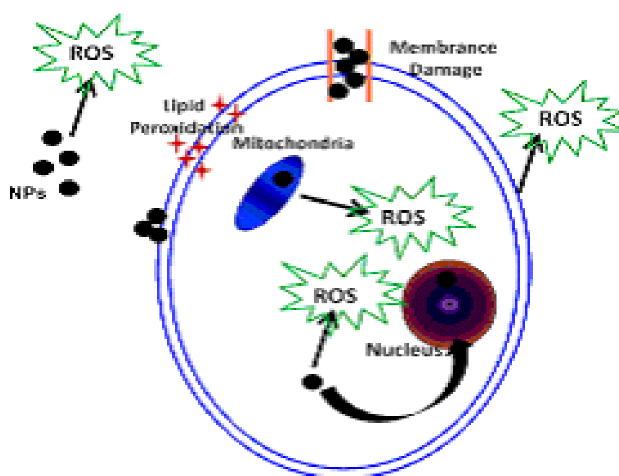


Fig.2: Proposed mechanism of interaction between TiO_2 NPs and cells

(Chan et al. 2011)

Based on this literature survey we came to know that nanoparticles have the ability to bind and interact with biological matter changing the surface characteristics depending on the environment they are present, resulting in an increased concern over its potential effect on human body. Amongst various types of toxicity studies done *in vitro* and *in vivo*, the DNA damaging effect or genotoxicity is the most important as it has sub-lethal effect on cells that can increase the rate of somatic mutations favoring carcinogenesis. The DNA damaging effect on germ cells can lead to heritable changes in the next progeny and thus poses major risk.

The genotoxic effect can be due to direct and indirect mechanisms. The direct method includes ROS generation and indirect method includes DNA binding and damage. Any compound with DNA binding activity can be considered as potentially genotoxic thus leading to concern for safety. The data for *in vitro* genotoxicity of nanoparticles are very limited. We propose to assess the genotoxicity of TiO_2 NPs in vitro using following experimental approaches.

1.4 IN VITRO GENOTOXICITY RISK ASSASSMENT

The term genotoxicity describes the property of chemical agents that damages the genetic information within a cell causing mutations, which may lead to cancer. It induces damage to the genetic material in the cells; it interacts with the DNA sequence and structure. The genetic alterations are detectable through assays that detect the substitution, addition, or deletion of nucleotides within a gene. Assays that measure gross structural chromosomal aberrations are those that detect breaks or chromosomal rearrangements involving one or more chromosomes and also numerical changes i.e. Aneuploidy.

We report our study with main focus on genotoxicity assay which was done using *in vitro* short term cultured Human peripheral blood lymphocyte chromosome aberration as per the OECD guideline no. 473. [4] The purpose of the in vitro chromosome aberration assay is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome and chromatid.

For testing of genotoxicity of TiO_2 NPs we have done two assays:

- Chromosome aberration assay
- Comet assay

1.5 DNA BINDING ACTIVITY WITH TiO_2 NANOPARTICLES

The genotoxic effect of a compound can be exerted mainly by DNA interaction.

Our study focuses on DNA binding activity of TiO_2 NPs. In comparison of bulk or non-Nano form of TiO_2, the NPs have higher surface to volume ratio which may make them more interactive and charged, that can bind with DNA. Interaction of TiO_2 NPs with human genomic DNA was analyzed by UV visible spectroscopy.

1.6 MICROSCOPY OF CELL CULTURES TREATED WITH TWEEN-80 COATED TiO_2 NPs

To check if the possible genotoxic effect involves cellular uptake, the TiO_2 NPs were coated with Tween 80 due to its auto-fluorescence property in order to enable microscopic observation. The Tween 80 is a surfactant which decreases the liquid to liquid and liquid to solid surface tension.

The current study was carried out with following aim:

- The safety concern related to biological effect of nanoparticles needs to be addressed
- We propose to study the *in vitro* genotoxicity as it is of major and long lasting concern
- The genotoxic potential of a compound can be due to direct and indirect effect on DNA. Hence DNA Binding activity, Cellular uptake and clasto-genicity will be assessed

Study design

- Study of clastogenicity of nanoparticles on metaphase chromosomes of short term cultured blood cells using chromosome aberration and COMET assay
- Study of *in vitro* DNA interaction with TiO_2 nanoparticles
  Culture of human whole blood with TiO_2 nanoparticles *in vitro* to assess the cellular uptake through fluorescence microscopy

MATERIALS AND METHODS

2.1 EQUIPMENTS

Autoclave	YORCO
Blood collection vials	B.D. BIOSCIENCE
Blood collection needles	GRENIAR BIO-ONE
Bench top Centrifuge	CM 101 REMI
Electrophoresis Unit	MAJOR SCIENCE
Gloves	KIMBERLEY CLARK
Glass ware	BOROSIL
Incubator	EIE INSTRUMENTS
Microscope	LABOMED
Microscope Slide	HIMEDIA, RIVERA
Sonicator	NINGBO HAISHU-SKLON
Slide box	TARSON
Quartz cuvette	SIGMA ALDRICH
UV- Visible Spectroscopy	AGILENT
Weighing Balance	SHIMADZU

2.2 REAGENTS

Acetic Acid	MERCK
Agarose	HIMEDIA
Chromic Acid	MERCK
Colchicine	HIMEDIA
Bromo phenol blue	THERMO SCIENTIFIC
EDTA Vacuette	BD BIOSCIENCES
EtBr	HIMEDIA
Ethanol	KUC
Giemsa Stain	SIGMA
Glacial Acetic Acid	MERCK
Methanol	MERCK
Mitomycin C	SIGMA
Potassium Chloride	MERCK
Qiagen DNA Blood MIDI kit	QAIGEN
RPMI-1640 culture media (AL,165A-50x10ml)	HIMEDIA
Sodium heparin Vacuette	BD BIOSCIENCES
Sodium hypo chlorite	MERCK
TrisCl	HIMEDIA
TiO_2 Nanoparticle (634662-25G)	SIGMA ALDRICH

2.3 REAGENT PREPARARTION

- **Culture Media:** RPMI-1640 (AL,165A-50x10ml) supplemented with 15% fetal calf serum, L-alanyl-L-Glutamine, HEPES buffer, Penicillin (60 mg/ liter), Streptomycin (100 mg/liter), and 2% $NaHCO_3$

- **KCl:** 0.56gm of KCl was dissolved in 100ml of sterile distilled water

- **Fixative:** Methanol and acetic acid were freshly mixed in 3:1 ratio respectively and chilled

- **Stock Giemsa stain:** 1gm Giemsa powder was added in 54ml glycerol, mixed and kept at 60°C water bath for overnight, 84ml of methanol was added, filtered, and kept in dark bottle

- **Working Giemsa stain:** 4ml Giemsa stain was added in 25ml Sorenson's buffer (pH 7) and 25ml water, mixed well and prepared freshly

- **Sorenson's buffer:** 0.345gm of monobasic sodium phosphate (NaH_2PO_4) and 0.454gm dibasic sodium phosphate (Na_2HPO_4) was added in 250ml of sterile distil water to obtain pH 7.0

- **Chromic acid:** 10% $K_2Cr_2O_7$ was added with 25% of H_2SO_4 for slide washing

- **Colchicine:** 10mg absolute powder was dissolved in 10ml of autoclaved deionized water and aliquots were dispensed (0.3mg/ml). It was used to block the cells at metaphase stage

- **TAE Buffer (Stock):** 24.2gm Tris base (100mM) in 100ml distilled water, 37.22 gm EDTA (10mM) in 100ml water

- **Working TAE Buffer:** 10mM Tris Cl and 1mM EDTA

- **EthBr:** 10mg EthBr per 1ml water for stock. Stored at -20°C in freezer

2.4 METHODS

2.4.1 CELL CULTURE

- **CHROMOSOME ABERRATION ASSAY**

In vitro chromosomal aberration test is to identify substances that cause structural chromosomal aberrations in cultured mammalian cell (OECD 473). Structural chromosome aberration or breakage is a missing, extra, or irregular portion of chromosomal. It can be in the form of atypical number of chromosomes or a structural abnormality in one or more chromosomes. The chromosome aberration test is most often performed on human peripheral blood lymphocytes. As peripheral lymphocytes are in the resting G0 stage of the cell cycle, they have to be stimulated to divide by a specific antigen, like phytohaemagglutinin. After 70th hours before fixation (at 70th hours) a spindle inhibitor like colchicine is added to block the cells in the (pro) metaphase of the second mitosis. Following hypotonic treatment the cell pellets were harvested stained and metaphase cells were analyzed microscopically for the presence of chromatid-type and chromosome-type aberration [4].

Primary aberrations are those seen at the first post-induction division, when all the parts are present and there has been no selection by passage though mitosis, or any modification by subsequent chromosome duplication (Savage, et al., 2016)

The majority of chemical agents which can induce aberrations, for ultra-violet light and most probably all “spontaneous” (and *de novo* aberrations) only primary chromatid-type are recovered. When, at subsequent interphase, the chromatids duplicate, surviving aberrations (and bits of aberrations) are converted into apparent chromosome-types, some of which are then transmitted almost indefinitely to further cell generations. These are the “derived” aberrations, and many are so modified that it is impossible to deduce their primary origin.

Thus, following an “acute” treatment with any clastogen, surviving cells in later generations carry only chromosome -type changes. The presence in such cells of chromatid-type aberration is, therefore, an indicator of an ongoing production of primary structural change, i.e. of some form of chromosome instability [7].

PROTOCOL

1.1 BLOOD SAMPLE COLLECTION

- Blood sample was collected from the healthy donor who was not exposed to antibiotics and other drugs, X-ray, infection or fever, etc in last three months
- Blood was aseptically collected in sodium heparin vacuates and mixed properly

1.2 NANOPARTICLES SUSPENSION PREPARATION (5mM STOCK)

STOCK SOLUTION: 10mg TiO_2 NPs in 25ml of autoclaved distilled water sample was sonicated for 10 min

- 200, 350 & 500 μM concentrations of TiO_2 NPs were added in three cultures

1.3 CULTURE SETTING PLAN

- Positive control (MMC)
- Negative control (only PHA)
- Three test concentrations

1.4 CULTURE SETUP

DAY 0

- Blood sample was collected in sodium heparin vacuates aseptically
- 1 ml blood was added in 10 ml RPMI-1640 (AL,165A-50x10ml) containing 15% fetal calf serum, L-alanyl-L-Glutamine, HEPES buffer, Penicillin (60 mg/ liter), Streptomycin (100 mg/liter), and 2% $NaHCO_3$
- Mixed well and incubated at 37˚C for 72 hours

DAY 1

- After 24 hours cultures were mixed well and incubated at 37˚C

DAY 2

- After 48 hours NPs at 3 concentration were added & mix well incubated at 37˚C

DAY3

- At 70th hour cultures were treated with colchicine (3μl); mixed well and incubated at 37˚C
- After completion of the 72 hours harvesting was done

CULTURE HARVESTING

1. Cultures were centrifuged at 3000 rpm for 10 min and supernatant was discarded
2. The pellet was resuspended in 0.56% KCl (Hypotonic solution) and incubated for 20 minutes at 37˚C
3. Slowly 4 – 5 ml of chilled fixative was added (3:1, methanol: glacial acetic acid)
4. Mixture was centrifuged for 10 minutes and the supernatant was discarded
5. Cell suspension was diluted in 4 – 5 ml of fixative and centrifuged
6. The step 2 and 3 were repeated, till the pellet became clear
7. Cell suspension was diluted in 1-2 ml of fixative
8. Few drops were dropped on the slides from height in presence of vapor for around 1 minute
9. The slides were labeled using a diamond marker pen
10. The slides were stained with 4% Giemsa for 5 min, rinsed in DW, and observed under the microscope using 10X and 100X
11. The slides were scored for aberration as per criteria by WHO and ISCN

SCORING OF CHROMOSOME ABERRATIONS

Counting of minimum 100 well-spread metaphases containing 46±2 chromosomes was done as per treatment group. The structural aberrations were scored in terms of gap, breaks, i.e. chromatid or chromosome type, acentric fragments, dicentric, exchange etc. were recorded on a score sheet.

Chromosome alterations are of two types:

- Structural chromosomal aberrations (chromosome or chromatid breaks, rearrangements)
- Numerical chromosomal aberrations (aneuploidy, polyploidy)

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids [4]

- Structural aberrations are of two types, chromosome and chromatid

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids [4]

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site [4] the chromosome aberration gap/break was seen rarely as compared to the chromatid break/gap.

2.4.2 Comet Assay

The COMET Assay is also done for detection of DNA damage where the damage of DNA is proportional to the length of tail in COMET.

PRINCIPLE

The comet assay is a simple method for measuring DNA strand breaks in eukaryotic cells.

The Single Cell Gel Electrophoresis assay (comet assay) involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline (pH-13) conditions, and electrophoresis of the suspended lysed cells.

Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. The likely basis for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. This is followed by visual analysis with staining of DNA and calculating fluorescence intensity to determine the extent of DNA damage. This can be performed by manual scoring or automatically by imaging software.

COMET is based on two principles:

- DNA migration is a function of both size and the number of broken ends of the DNA
- Tail length increases with damage initially and then reaches a maximum that is dependent on the electrophoretic conditions, not the size of fragments

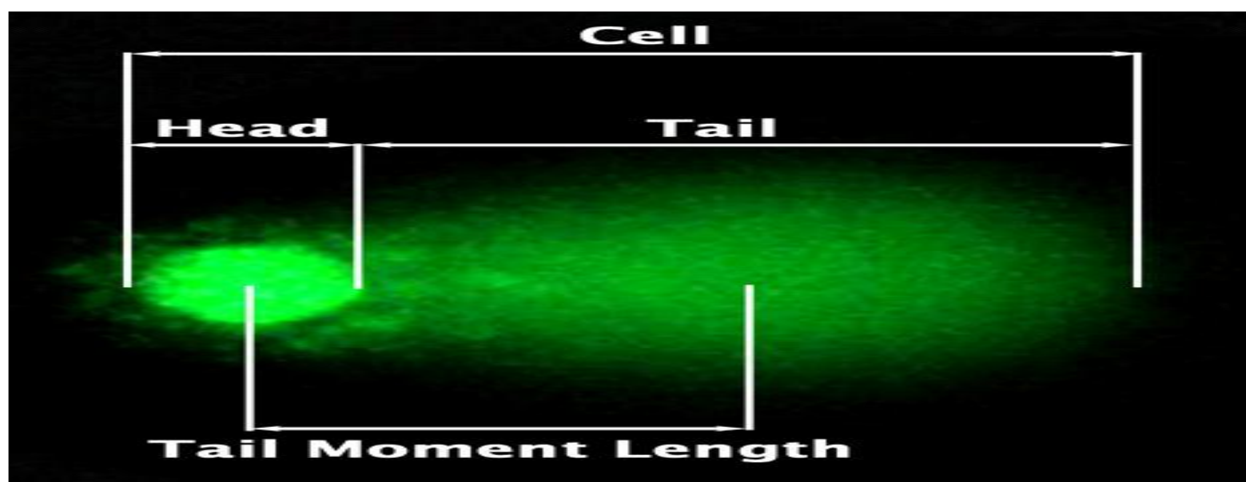


Fig 3: A typical COMET analysis depicting a cell with head, tail and background regions of interest (cometassayindia.org)

PROTOCOL

- Base layer was prepared on clean slide by dipping it in 1% NMP (Normal melting point agarose) agarose (1gm agarose in 100mL of distilled water)
- Second layer was prepared by mixing 10μL of sample with 90μL of 1% LMPA (Low melting point agarose, 1gm of LMPA in 100mL of distilled water) and then it was overlaid on base layer and with the help of coverslip second layer is prepared
- Than slides were kept at 4˚C for 10 min for gel solidification after that coverslip is removed
- At last third layer was prepared of 1% LMPA over the second layer with the help of coverslip
- It was allowed to solidify for 10 min at 4˚C and then coverslip was removed
- Then slides were kept in pre-cooled lysis solution(2.5M Nacl,100 Mm EDTA,10mM Tris, 10% DMSO, 1% Triton X-100, 10% N-Lauryl Sarcosine, 10mM DTT [pH-10]) for 24 hours
- After that slides were washed with 1X PBS solution and kept in unwinding buffer (300mM NaOH,1mM EDTA) [pH-13] for 30 min and then washed with 1X PBS solution
- Slides were kept in electrophoresis chamber and electrophoresis was then carried out at 24 V for 1 hours in 1X TBE buffer
- After that slides were remove and washed with 1X PBS and stained with EtBr for 5 min and then washed with 1X PBS and observed under fluorescence microscope
- Images were captured and analyzed by comet score software

COMET assay measurement:

Olive Tail Moment: Tail moment is defined as the product of tail length and the fraction of the total DNA in the tail. Tail moment incorporates a measure of the both the smallest detectable size of migrating DNA and the number of broken pieces.

% DNA in tail and head: The total fluorescence intensity within the delineated comet head is compared to the total comet intensity within the region of interest to determine the percentage of DNA in the head.

Tail length: The tail length has been defined as the distance between the center of the comet head and the last non-zero pixel of the comet profile. In our case the profile in the x-direction is used and corrected for comet angle.

2.4.3 DNA BINDING ACTIVITIES OF TiO_2 WITH UV SPECTROPHOTOMETER

ISOLATION OF GENOMIC DNA FROM HUMAN BLOOD (QAIGEN DNA BLOOD MIDI KIT)

PROTOCOL

- Pipette out 200µl protease into the bottom of a 15 ml centrifuge tube
- 1-2 ml of blood was added & mixed briefly
- 2.4 ml AL buffer was added to sample & mixed by vortex for 15 min
- The mixture was Incubated at 37˚ C for 10 min
- 2 ml Ethanol was added to the sample tube, mixed 10 times inverting the tube
- Transfer the solution in midi column placed in a 15 ml centrifuge tube without wetting the rim, the cap was close and Centrifuged at 3000 rpm for 3 minute
- The midi column was removed, discarded the filtrate and Placed the spin column in a clean 15 ml collection tube
- Without moistening the rim added 2 ml AW1 buffer to the midi column and centrifuged at 5000 rpm for 1min
- 2 ml AW2 buffer was added to the midi column and centrifuged at 5000 rpm for 15 min
- The midi column was transferred in a clean 15 ml centrifuge tube & the filtrate was discarded
- 300 µl AE buffer or D/W was added to the midi column, equilibrated to room temperature for 5 min and centrifuged at 5000 rpm for 2 min
- Collected the filtrate in micro centrifuge tube

2.4.4 AGAROSE GEL ELECTROPHORESIS

PROTOCOL

- The edges were sealed of a clean, dry glass plate with tape to form a mold
- TAE buffer was prepared to fill the electrophoresis tank and to cast the gel
- A solution of 1% Agarose was then prepared in buffer for separating the particular size fragments in the DNA sample
- The slurry was heated in a microwave oven until the agarose melted
- When the melted gel was cooled, 3µl/ml ethidium bromide was added into gel
- The gel solution was mixed thoroughly by gentle swirling
- While the agarose solution was cooling, chosen an appropriate comb for forming the sample slots in the gel
- The agarose gel was poured into the mold plate
- The gel was allowed to solidify completely, the comb was remove carefully and then buffer was poured on the one edge of casting plate to top of the gel, The electrophoresis buffer was poured off and the tape was removed carefully and The gel was mounted in the electrophoresis tank
- TAE buffer was added to cover the gel

- The samples of DNA were mixed with 2µl of loading dye
- Slowly the sample mixture was loaded into the slots of the submerged gel using a micropipette
- The lid of the gel tank was closed and electrical leads were attached and electrical current was applied so that the DNA would migrate towards the positive anode (DNA having negative charge)
- When the DNA samples had migrated a sufficient distance through the gel, electric current was turned off and the lid was removed from the gel tank
- Examine the gel by UV light and photograph the gel

2.4.5 SPECTROPHOTOMETER

Spectrophotometer is an analytical instrument used in molecular biology to determine the average concentration of the nucleic acid DNA or RNA present in a mixture, as well as their purity.

PROTOCOL

- The upper and lower optical surface of micro spectrophotometer sample retention system was cleaned
- NanoDrop software was opened and selected the Nucleic acid module
- Performed a blank measurement by loading 1 µl deionized water or buffer and selected “blank”
- 1µl nucleic acid sample was loaded and selected “measure”
- Once the measurement was completed, both the optical surface was cleaned
- The concentration and purity of nucleic acid was noted in terms of ratio of absorption at $_{260/280nm}$

PRINCIPLE

UV VISIBLE SPECTROPHOTOMETER:

Ultraviolet spectroscopy is a technique used to quantify the light that is absorbed and scattered by a sample.

In its simplest form, a sample is placed between a light source and a photo detector, and the intensity of a beam of light is measured before and after passing through the sample.

These measurements are compared at each wavelength to quantify the samples wavelength dependent extinction spectrum.

The data is plotted as extinction as a function of wavelength. Each spectrum is background corrected using a blank, a cuvette filled with only the dispersing medium (water) to confirm that spectral features from the solvent are not included in the sample extinction spectrum.

Nanoparticles have optical properties that are sensitive to size, shape, concentration, agglomeration state, and refractive index near the nanoparticle surface, which makes

UV/Vis/IR spectroscopy a valuable tool for identifying, characterizing, and studying these materials.

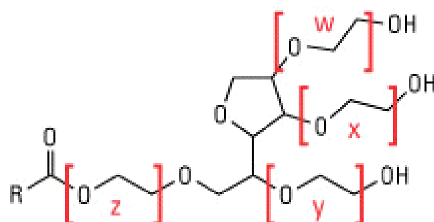
The interaction of TiO_2 nanoparticles with human genomic DNA has been studied with UV–visible spectroscopy in order to investigate the possible binding of nanoparticle to DNA.

PROTOCOL

- The Human Genomic DNA was isolated from normal WBCs of peripheral blood using the protocol of Qiagen's kit
- Quantification of DNA by NanoDrop and Agarose Gel Electrophoresis
- UV absorption spectra of human genomic DNA was studied at 259 nm
- Addition of various concentration of TiO_2 nanoparticles
- The spectrum was measured with Agilent Carry-60 UV visible spectrophotometer
- Spectral changes of 30.2 µg/ml DNA were monitored after adding various concentrations of TiO_2 NPs (25µM-250µM) by recording the UV–visible absorption (200–800 nm)
- All experiments were run in Tris-base buffer (0.1M), pH 7.5, and 37 °C
- The recording chart read temperature and absorbance differences between the reference cuvettes
- The DNA and DNA with TiO_2 Nanoparticles at increasing concentrations were noted

2.4.6 STUDY OF CELLULAR UPTAKE OF NANOPARTICLES COATED WITH TWEEN-80 USING FLUORESCENCE MICROSCOPY

Tween-80 is a popular non-ionic surfactant used widely in cosmetics, foods, pharmaceutical products and biochemical research. It decreases liquid to liquid or liquid to solid surface tension.



Tween* 80 Detergent

$w + x + y + z = 20$

$R = CH_2(CH_2)_5CH_2CH{=}CHCH_2(CH_2)_6CH_3$

MW 1310

PROTOCOL

- 50 mg of TiO_2 NP was mixed in 50 ml distilled water
- The suspension was sonicated with 400V, at 25˚C, for 10 min
- Suspension was vigorously stirred on magnetic hot plate at 56˚C for 30 minutes
- After completion of 30 minutes, 4-5 ml tween 80 was added into the suspension and again the suspension was placed on magnetic stirring hot plate at 50˚C for 2 hours
- After completion of 2 hours, the suspension was transferred in centrifuge tube and centrifuged at 3000 RPM for 10 min
- Supernatant was collected and the pellet was dispersed in water and the solution was mixed completely
- Repeated 6,7 steps for three times
- The OD was taken between the range of 200-800 nm with the help of UV spectroscopy
- The wavelength difference between coated NPs versus uncoated NPs was checked
- Then the coated NPs were checked under fluorescence microscopy under blue filter
- Then for the cellular uptake culture set up was done for 24 hours
- Harvesting was done without using hypotonic solution
- Cells were observed under fluorescence microscopy

RESULTS AND DISCUSSION

3.1 CHROMOSOME ABERRATION ASSAY (Results of experiment repeated three times)

Experiment: 1

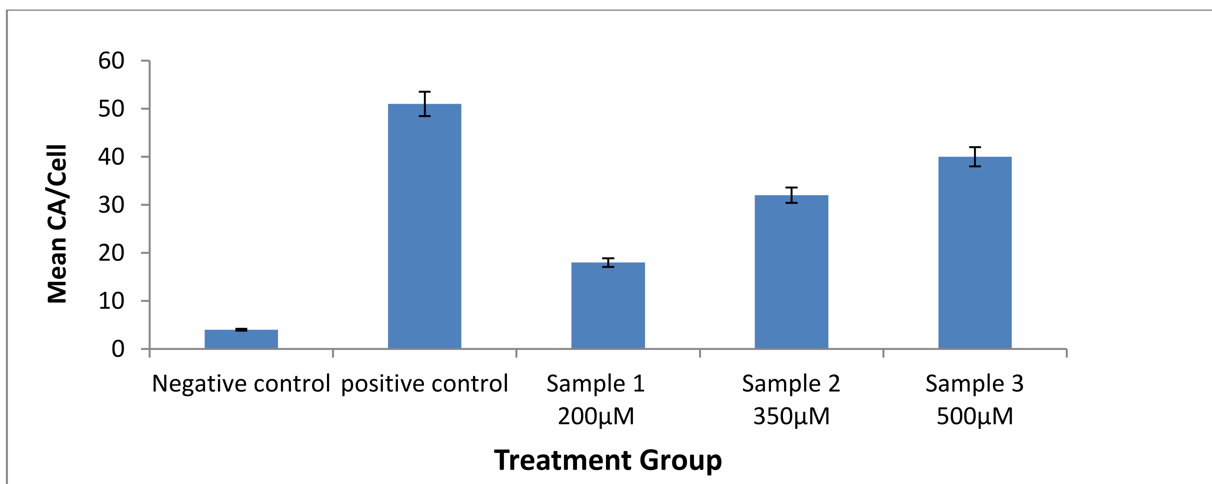
Table 1.1A Frequency of chromosome aberrations found in all treatment groups

No.	Treatment Group	Frequency of chromosome aberrations in 100 cells
1	Negative control	04
2	positive control	51
3	Sample 1 (200µM)	18
4	Sample 2 (350µM)	32
5	Sample 3 (500µM)	40

Treatment Group	Mean	Std. Error	T value	P value
Sample 1 (200µM)	0.18	0.0384	3.25	*
Sample 2 (350µM)	0.32	0.0466	5.54	**
Sample 3 (500µM)	0.40	0.0439	6.83	***

(*P<0.05; **P<0.01; ***P<0.001)

Table 1.1B Effect of TiO_2 NPs on mean CA/cell values of *in vitro* short term cultures of peripheral blood lymphocytes



Graph 1: Graphical representation of results of chromosomal aberration assay

Experiment: 2

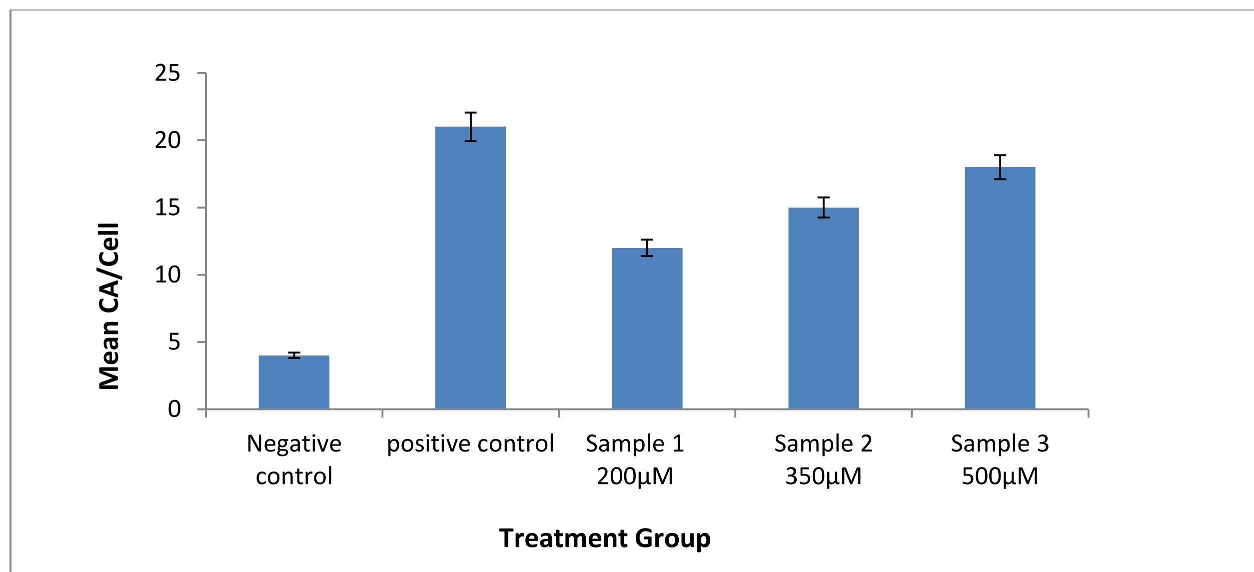
Table 2.1A Frequency of chromosome aberrations found in all treatment groups

No.	Treatment Group	Frequency of chromosome aberrations in 100 cells
1	Negative control	04
2	positive control	21
3	Sample 1 (200µM)	12
4	Sample 2 (350µM)	15
5	Sample 3 (500µM)	18

Treatment Group	Mean	Std. Error	T value	P value
Sample 1 (200µM)	0.12	0.0324	2.11	*
Sample 2 (350µM)	0.15	0.0357	2.70	**
Sample 3 (500µM)	0.18	0.0384	3.85	***

(*P<0.05; **P<0.01; ***P<0.001)

Table 2.1B Effect of TiO_2 NPs on mean CA/cell values of *in vitro* short term cultures of peripheral blood lymphocytes



Graph 2: Graphical representation of results of chromosomal aberration assay

Experiment: 3

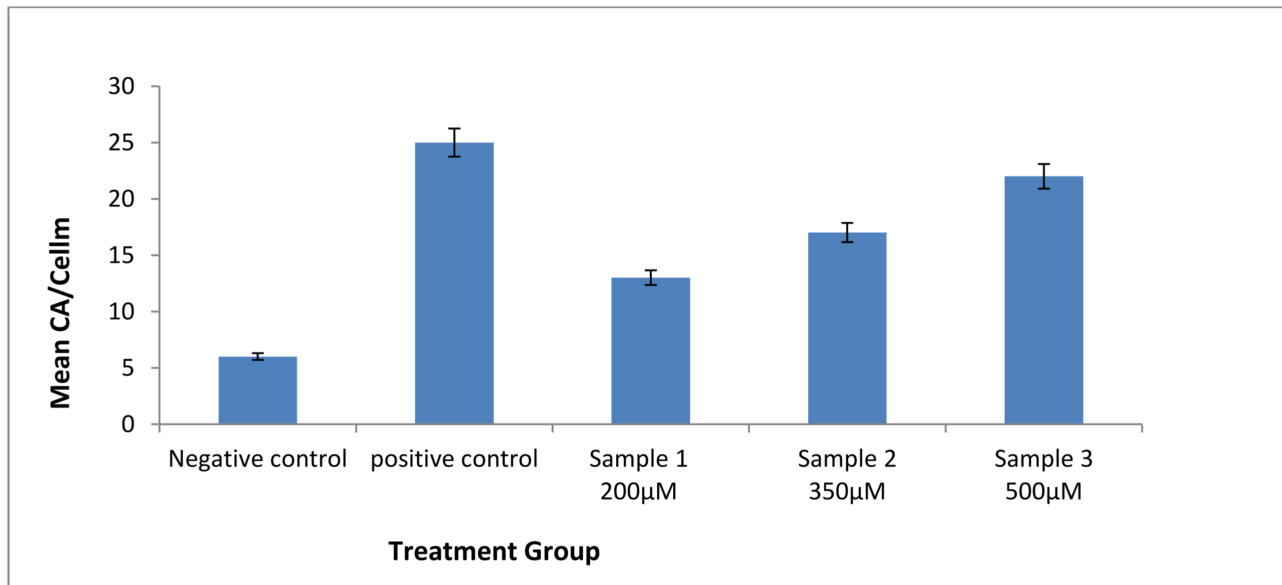
Table 3.1A Frequency of chromosome aberrations found in all treatment groups

No.	Treatment Group	Frequency of chromosome aberrations in 100 cells
1	Negative control	06
2	positive control	25
3	Sample 1 (200µM)	13
4	Sample 2 (350µM)	17
5	Sample 3 (500µM)	22

Treatment Group	Mean	Std. Error	T value	P value
Sample 1 (200µM)	0.13	0.0336	1.70	*
Sample 2 (350µM)	0.17	0.0375	2.47	**
Sample 3 (500µM)	0.22	0.0414	3.35	***

(*P<0.05; **P<0.01; ***P<0.001)

Table 3.1B Effect of TiO_2 NPs on mean CA/cell values of *in vitro* short term cultures of peripheral blood lymphocytes



Graph 3: Graphical representation of results of chromosomal aberration assay

The possible genotoxic effect of TiO_2 NPs was evaluated by chromosome aberration assay using short term cultured human blood cells following in vitro exposure to various concentrations (200μM, 350μM, 500μM) of TiO_2 NPs. The results of groups treated with TiO_2 NPs showed increase in the chromosomal aberrations. Treatment with all the three concentrations of TiO_2 NPs suspension for 24hrs induced significant number of structural chromosomal aberrations in dose dependent manner. The aberration rates at all the tested concentrations of TiO_2 NPs suspensions were higher than the control, suggesting the possible clastogenic activity of NPs.

Table no. 1.1A, 1.1B, 2.2A, 2.2B, 3.1A, 3.1B indicate significant increase in number of structural chromosomal aberrations following exposure to above concentrations of the TiO_2 NPs. The aberration rates at all the tested concentrations of the NPs were higher than the control (significant P value using Student's t-test) suggesting possible clastogenic activity of the TiO_2 nanoparticles.

The mechanism of genotoxic effect in terms of induced frequency of chromosomal aberrations can be direct or indirect; i.e. by direct DNA interaction and by inducing ROS that can damage DNA.

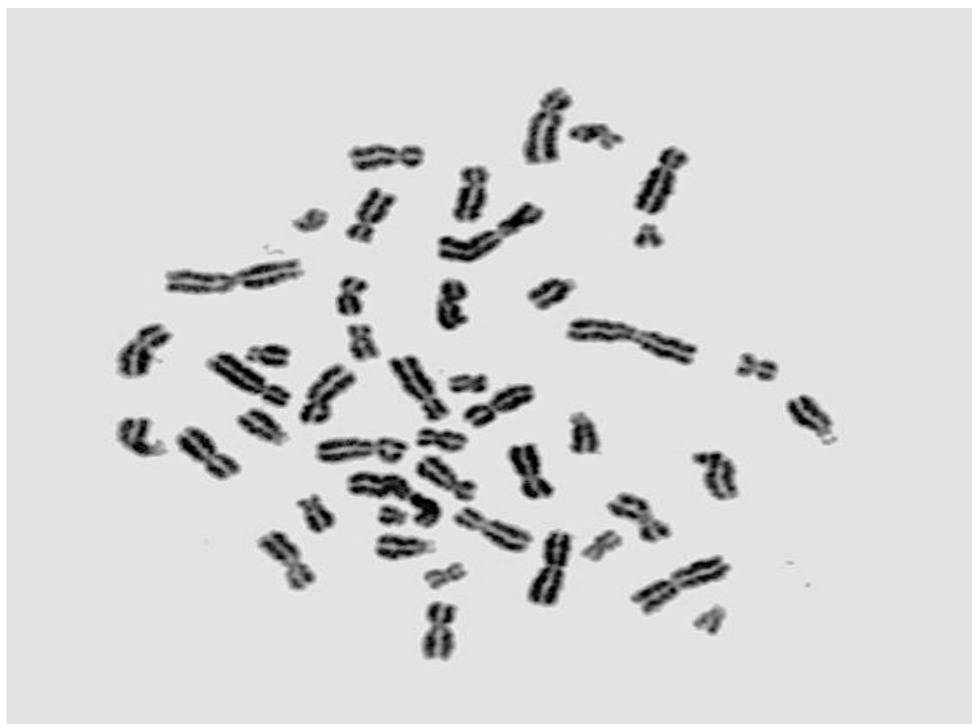


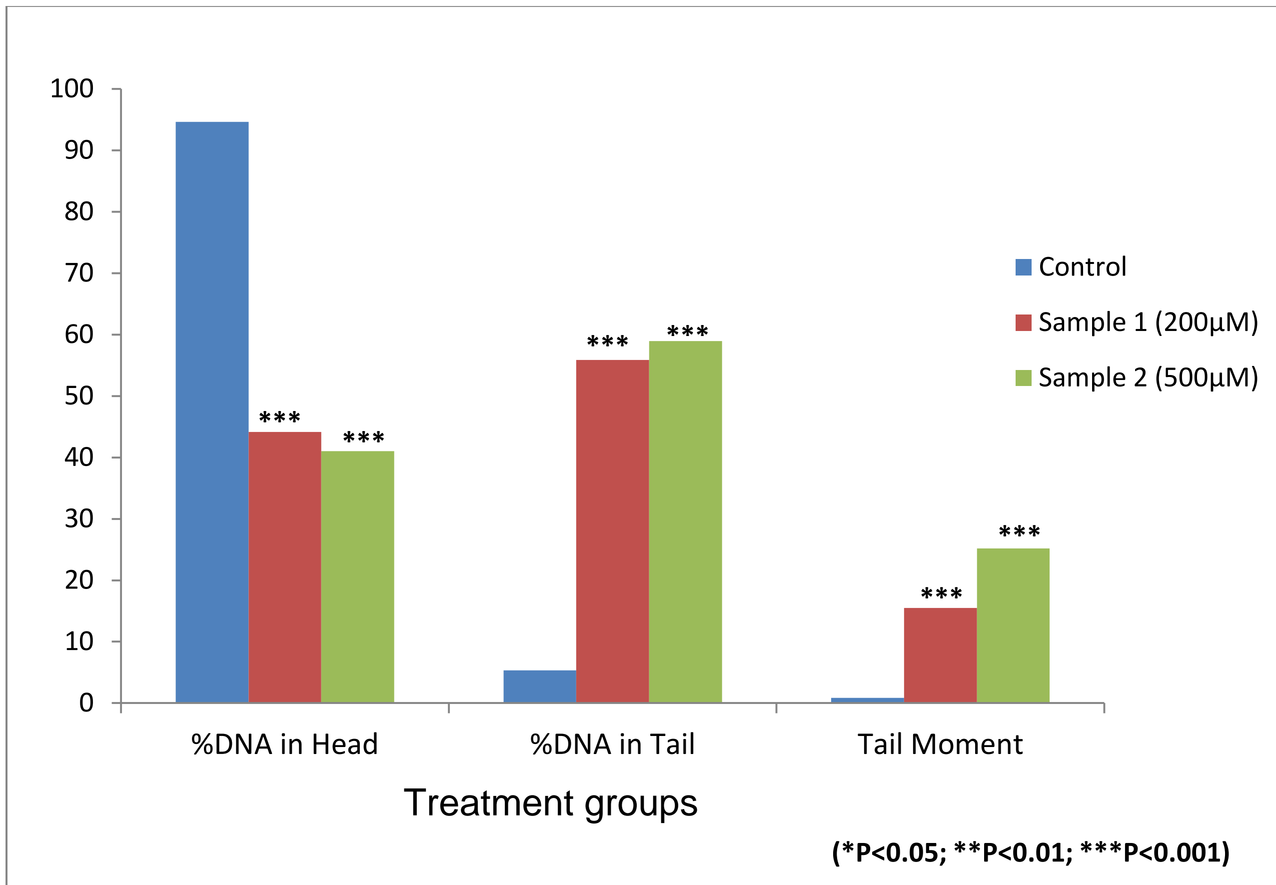
Fig. 4(A): A representative photomicrograph depicting Metaphase Chromosome plate without structural aberration (Magnification: 1000X with digital zoom)

Fig. 4(B): A representative photomicrograph, small arrow depicting chromatid break and big arrow depicting chromatid gap (Magnification: 1000X with digital zoom)

3.2 COMET ASSAY

Table 3.2.1; Shows the detail about %DNA in Head, %DNA in Tail, Tail Moment of treatment groups

Group	%DNA in Head	%DNA in Tail	Tail Moment
Control	94.66	5.34	0.86
Sample 1 (200µM)	44.13	55.87	15.49
Sample 2 (500µM)	41.03	58.97	25.19



Graph 4: Graphical representation of COMET assay

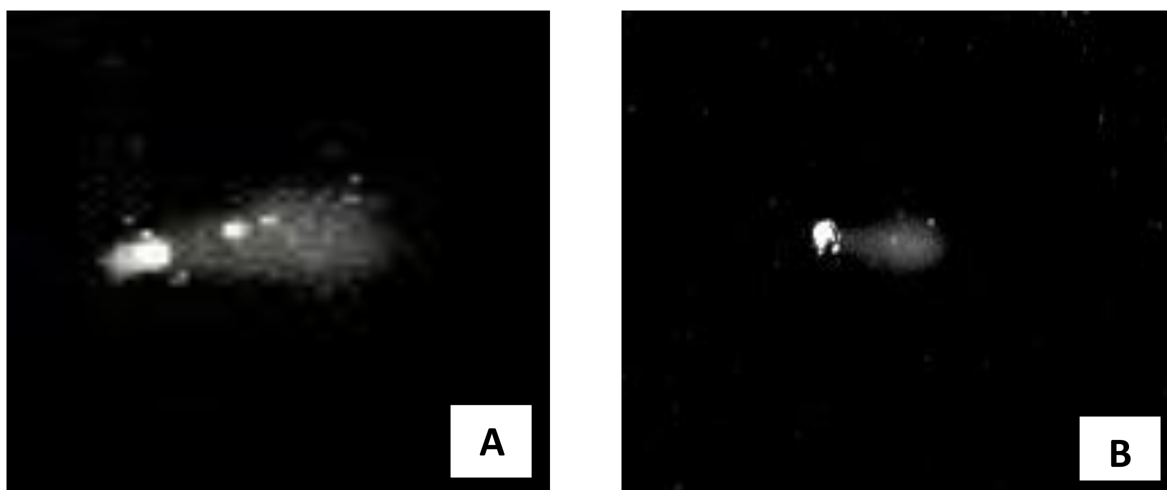


Fig.5: A representative photomicrograph depicting results of comet assay in cell cultures treated with A) TiO_2 (200μM) & B) TiO_2 (500μM);
[Magnification: 1000X with digital zoom]

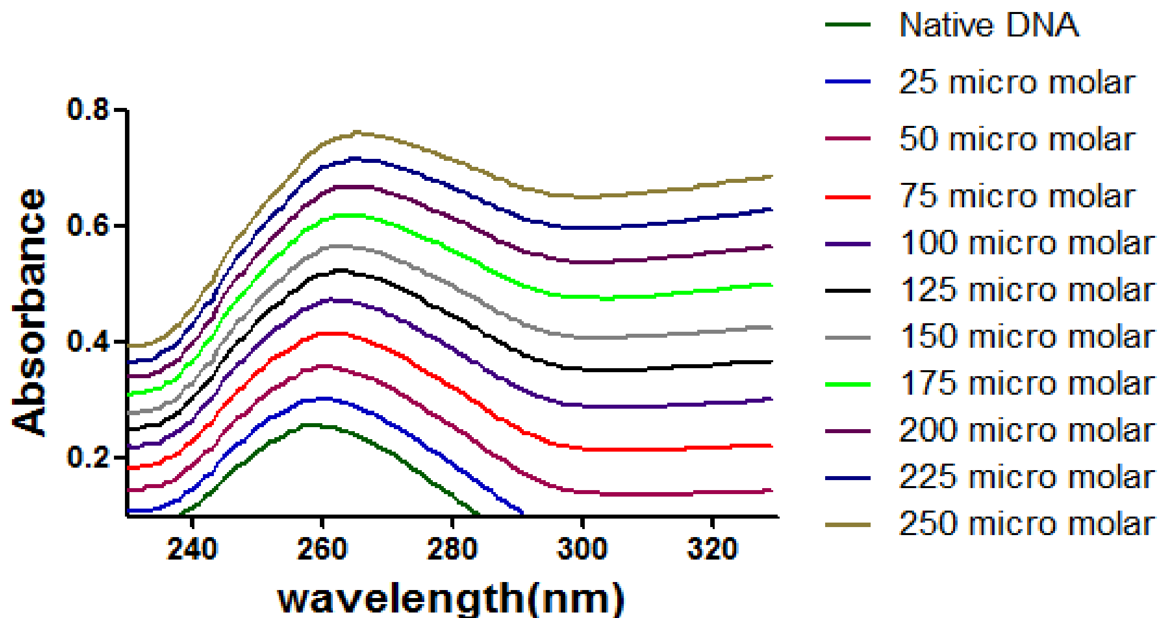
The Chromosome Aberration (CA) assay represents effect on metaphase cells where the extent of damaged DNA in interphase cells is not considered. In order to assess this, COMET assay is carried out which measures DNA breakage in non-dividing cells. Thus both the assays together give an idea about clastogenic effect of NPs on dividing and non-dividing cells.

The DNA damage has been checked by COMET assay using short term culture method with various concentrations (200μM, 500μM) of TiO_2 NPs. Here, in this assay we are considering three different parameters which are % DNA in head, % DNA in tail and tail moment.

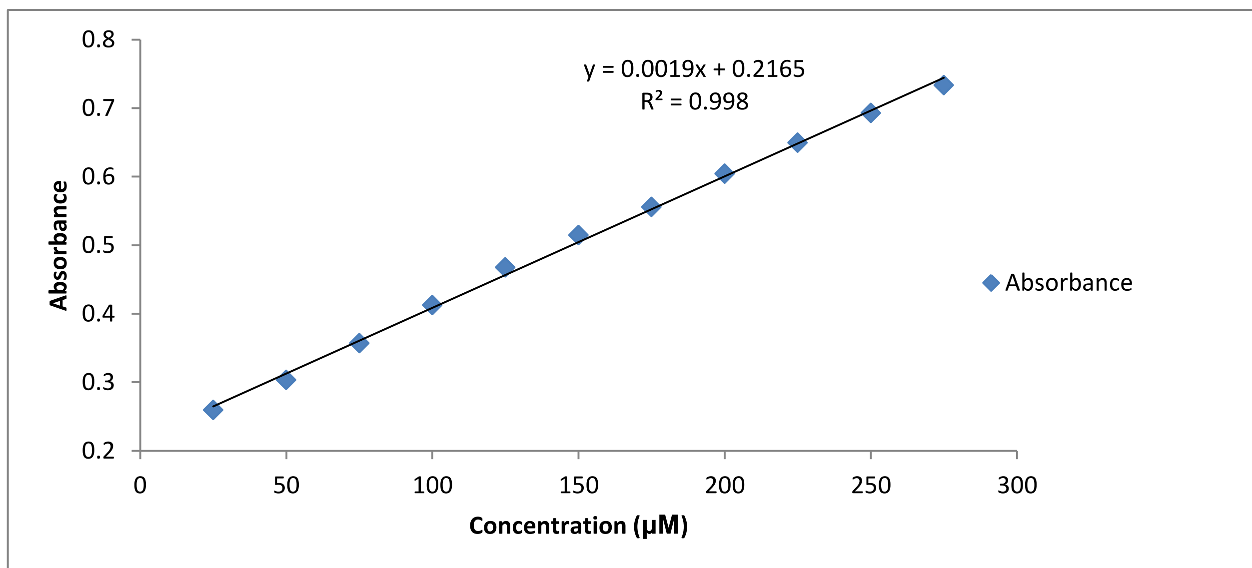
The graph 4 depicting the % DNA in head shows the % DNA which is not damaged. It is higher in the control group rather than the treatment group, which shows less damage in DNA in control group in comparison with treatment group. The % DNA in tail means the % of DNA is damage. It is less in control group in comparison with the treatment group, which represents the higher damage in the treatment group. The tail moment shows the product of the tail length and the fraction of total DNA in the tail which is lesser in the control group rather than the treatment group.

The study of all these parameters helps us to know about the DNA damage induced by the treatment of TiO_2 NPs. The result indicates the significant increase in damage with increase in treatment of TiO_2 NPs. The significant P value was obtained by using one way ANOVA test.

3.3 DNA BINDING ACTIVITY WITH TiO_2 NANOPARTICLES



Graph 5: Changes of UV spectra of human genomic DNA in the presence of different Concentrations of TiO2 nanoparticle in Tris-base buffer (0.1 M), pH 7.5 at 37 °C.



Graph 6: Alterations in maximum absorbance (at 259 nm) of human genomic DNA in the presence of different concentrations of TiO2 nanoparticle

UV visible studies

Interaction of DNA with TiO_2 NPs is studied by recording the absorbing spectra for a constant DNA concentration in various TiO_2 NPs mixing ratio at 37°C, the results of which are shown in Fig 1. Absorption spectroscopy helps to determine the binding activity of metal complexes with DNA.

The spectra changes observed in the form of "**Hyperchromism"** and "**Hypochromism"** during the process reflect the change in conformation of DNA and structure of DNA. Titanium dioxide can be bind to DNA via covalent or non-covalent interactions. DNA base pairs have strong optical absorption at 260 nm.

The absorption spectra of DNA in absence and presence of TiO_2 Nanoparticles is shown in Fig1(a) with addition of increasing amount of TiO_2 nanoparticles to DNA, there is an increasing in absorbance was observed in the maxima wavelength of 259 nm.

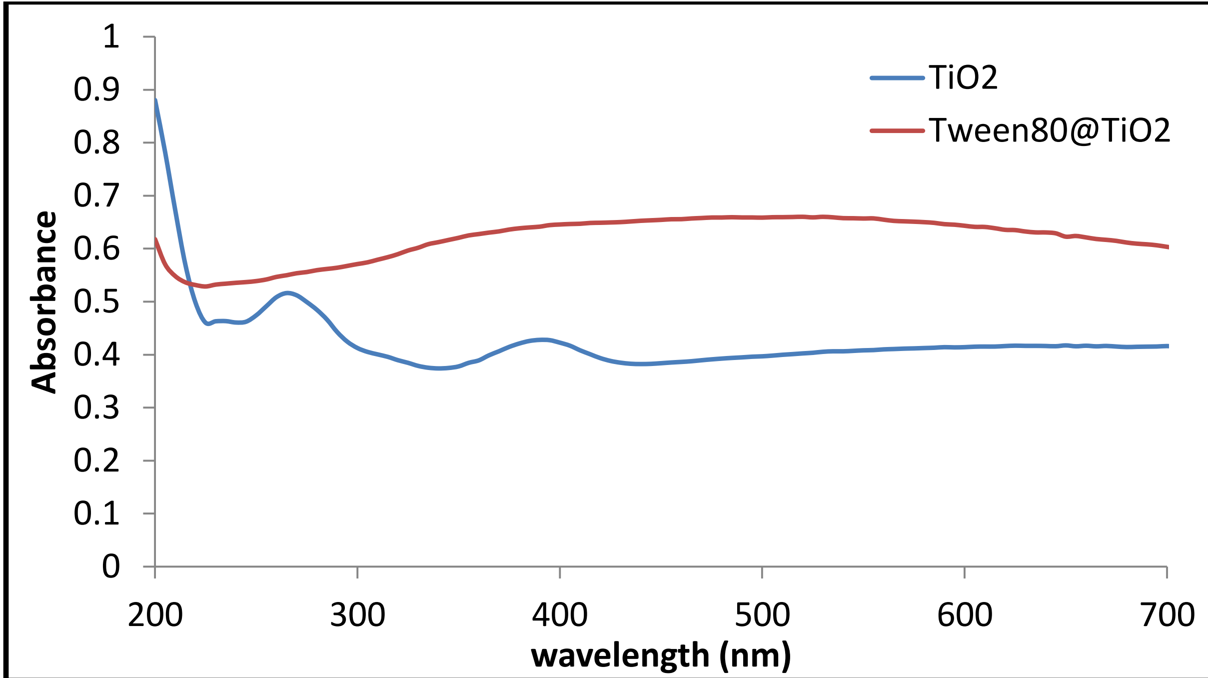
Hyperchromism is reported to suggest breakage of the secondary structure of DNA and therefore this hyper chromic effect show there strong interaction between nanoparticles and DNA.

The DNA binding results of which are shown in Fig 1(a).The metal complex can bind to DNA via covalent and or/non-covalent interactions. Non-covalent DNA interacting agents can change DNA conformation, change DNA torsional tension, and potentially lead to DNA strand breaks, which is one of the end point of genotoxicity.

We hypothesize various modes in which the nanoparticles bind to DNA, resulting in an increase in absorbance of NPs.

- The absorption intensity at 259 nm is increased because exposure of purine and pyrimidine bases of DNA when TiO_2 NPs bind to DNA, result in slight change in the conformation of DNA
- In presence of electrostatic interaction between TiO_2 NPs and DNA, conformational and structural changes take place in DNA
- Addition of TiO_2 NPs results in base interaction being reduced in DNA, due to which many bases would be free form and H-bonding is not present between complementary bases. This can lead to an increase in UV absorbance
- Hyperchromism can also be due to the external contact or partial uncoiling of the helix structure of DNA, exposing more bases of the DNA

- **3.4 MICROSCOPY OF CELL CULTURES TREATED WITH TWEEN-80 COATED TiO_2 NPs**



Graph 7: UV-Vis spectra shows difference in absorbance of uncoated Tio_2 and tween 80 coated TiO_2

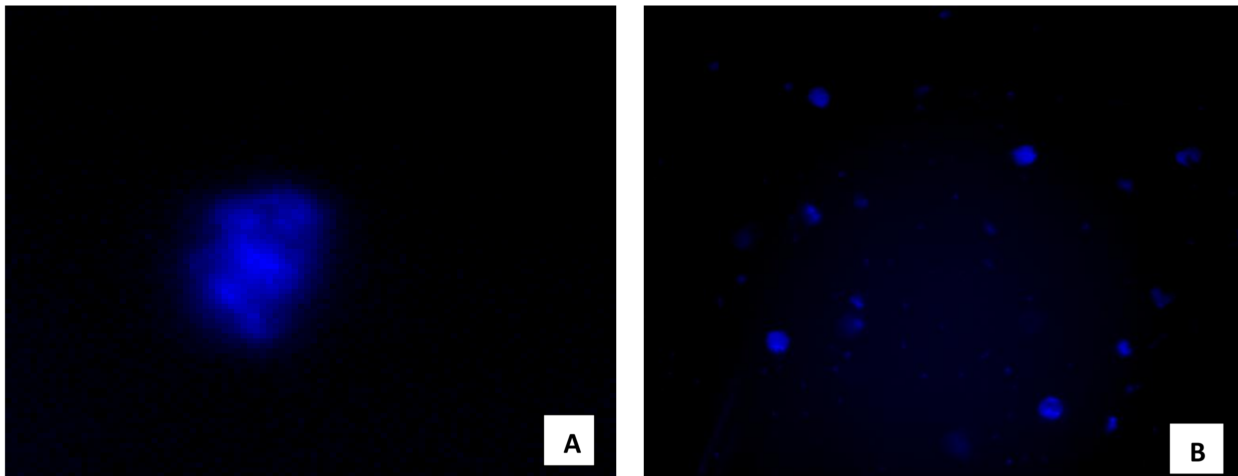


Fig.6: Fluorescence microscope image using DAPI filter(478-495nm), of (A) Tween-80 coated TiO2 NP & (B) lymphocytes incubated in the presence of Tween-80 coated TiO_2 NP depicting auto fluorescence of NP alone and no intracellular uptake under current experimental condition

The possibility of direct mechanism can be studied by; 1) DNA binding activity of NPs using spectrophotometry and 2) observing internalization of NPs in the cultured cells in vitro. We carried out Tween-80 coated NPs cellular uptake through using short term culture method.

Initially UV spectrum was taken to differentiate the coated and uncoated NPs. In which we obtained the absorption difference between both of them. That indicates the coating of TiO_2 NPs.

The fluorescence microscopy of NPs without cellular uptake shows the fluorescence under the DAPI filter (478-495nm), which also indicates the coating of NPs.

For the cellular uptake of coated TiO_2 NPs, fluorescence microscopy was done, but it didn't show the presence of coated TiO_2 NPs inside the cells. The culture was set for 24 hours, then also the cellular uptake was not observed so, it might be because of the less time exposure to the TiO_2 NPs.

SUMMARY

Nanotechnology has attracted considerable attention in the scientific community ever since its emergence as a powerful basic and applied science tool. Nanoparticles have many special physicochemical properties, and thereby may contribute to extraordinary hazards for human health and the environment. The current major applications of TiO_2 nanoparticles includes; selfcleaningcements, glass and paints; water purification systems; antifogging coatings for glass; and as a UV attenuating ingredient in lotions, sunscreens and cosmetics. The unique properties of TiO_2 nanoparticles may alter the way they interact with biological molecules and consequently, their toxicity. According to IARC guideline the TiO_2 NPs are classified under group 2-B, which means possibly carcinogen. The genotoxic effects of any particle could be of two types: Direct and Indirect. Direct method includes the ROS generation and the indirect method includes DNA binding.

These all applications lead to the concern about the genotoxic effects of the TiO_2 NPs. Here we aimed to study the clastogenicity of nanoparticles on metaphase chromosomes of short term cultured blood cells using chromosome aberration and COMET, the *in vitro* DNA interaction with TiO_2 nanoparticles, the human blood cells with TiO_2 nanoparticles *in vitro* and assess the cellular uptake through fluorescence microscopy

The present study focuses on In vitro assessment of genotoxicity of Titanium Dioxide Nanoparticle by human cultured peripheral blood cells in terms of Chromosomal Aberration assay. The purpose of the in vitro chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome or chromatid.

We aimed to check the genotoxicity risk assessment for TiO_2 NPs. For that we had done two different assays: chromosome aberration assay, in which we got the dose dependent increase in aberration, and second COMET assay which is for checking DNA damage, in that we got significant increase in DNA damage than the control group.

In case of DNA and TiO_2 NPs binding, the uv spectrophometer was done. In that we got the native DNA at 259nm. After addition of TiO_2 NPs with 25 to 100 (micromolar concentration), it indicated hyperchromicity (increase in absorption) with increase in the concentration of TiO_2 NPs. That indicates the change in the complex as the TiO_2 binds with DNA.

For checking of cellular uptake of TiO_2 NPs, we had coat them with tween 80, which is a surfactant with auto fluorescent properties. The coated NPs were checked under UV spectrophotometer which also had given us different absorption and fluorescence

microscopy had given us fluorescence under blue filter. But after culture set up of 24 hours, and harvesting, the cellular uptake was not observed under the microscope. It could be because of less time incubation of the culture.

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APPENDICES

SOP (Standard Operating Procedure)

6.1 MICROSCOPE:

1. Remove the dust cover.
2. Switch on the microscope.
3. Clean-dry the slide if wet, clean the stage.
4. Check the optical lens, clean if required with tissue paper only.
5. Check Kohler illumination, reset required.
6. Start with 4X, move to higher magnification clockwise only.
7. When using higher magnification adjust stage height to avoid impact on objective lenses.
8. If using 100X objective, use very little amount of oil and avoid 40X touching the oil.
9. Clean the stage and optical lenses with tissue paper.
10. First lower the light and switch off.
11. Put the dust cover back.

6.2 HIGH SPEED COOLING CENTRIFUGE:

1. Switch on mains. Switch on power button of Instrument. Wait till screen starts.
2. Press ‘SET’. It will ask for password (i.e. 000).Press enter three times by pressing rotational knob.
3. Pressing of this knob will work as enter and rotating will serve as up and down.
4. Check Specification before the setting your Programme.
5. Set Programme No., head no, acceleration, deceleration, RPM, Temperature and timer sequentially.
6. Press SAVE to save Programme.
7. Put eppendrof / tube such that rotor is balanced.
8. Close the lid of rotor properly.
9. Close lid of instrument properly. Centrifuge will not start unless lid is closed.
10. Press Start to run centrifuge. A smooth whirring should be heard. In case of load or unusual sound press stop immediately to terminate run and report the problem.

6.3 U.V Visible Spectrophotometer:

1. Turn ON the instrument and computer from the main.
2. Start CPU and monitor.
3. Start Agilent power button. It will blink red light.
4. It will turn into green. If it not turns into green immediately report.
5. Operate from computer. Click on Carry win.
6. Select your required application.
7. Click on Set UP. Set your desire wavelength.
8. Put Blank tube in sample holder. Click on Zero.
9. Put Samples one by one and click on Read.
10. Note down your readings. Do not save window of simple read.
11. Turn OFF the instrument power button, than shut down computer, then switch OFF mains.

6.4 CHROMOSOME ABERATION ASSAY SCORE SHEET

1	2	3	4	5	6	7	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75	76	77	78	79	80
81	82	83	84	85	86	87	88	89	90
91	92	93	94	95	96	97	98	99	100

Metaphase No	Vernier Reading	Chromosomes		Chromatids		Others aberration
		Break	Gaps	Break	Gaps	

Total Aberrations count: