Chapter 3
Materials and Methods
MATERIALS AND METHODS

3.1. Test Animals

3.1.1. Species and Strains

Wistar rats were used in the present study. The choice was usually be determined by cost, availability and convenience.

3.1.2. Sex

The present study was carried out in male Wistar rats. Extensive studies of the activity of known clastogens in the mouse bone marrow micronucleus test have shown that in general male rats are more sensitive than female rats for micronucleus induction. A detailed collaborative study was carried out indicating that in general male rats were more sensitive than female rats for micronucleus induction, but where differences were seen they were only quantitative and not qualitative (The Collaborative Study Group for the Micronucleus Test, 1986). This analysis has been extended by the group considering the micronucleus test at the International Workshop on Standardisation of Genotoxicity Procedures, Melbourne, 1993 and having analysed data on 53 in vivo clastogens (and 48 non-clastogens), the same conclusions were drawn (Hayashi et al., 1984).

3.1.3. Weight

Adult male Wistar rats weighing 180-200 g b.w. were used in this study.

3.1.4. Age

There is generally a higher mitotic index in younger animals and so it is suggested that animals that have reached sexual maturity be used (e.g. 10-12 wk for rat), and that those of advanced age be avoided.

3.1.5. Housing

The animals were maintained on standard laboratory condition i.e.; room temperature of 18 ± 25°C; relative humidity 45-55%, 12:12h light/ dark cycle and given food and water ad libitum. The animals were made to acclimatize with the laboratory conditions for a period of 15 days in a healthy condition prior to study. The whole experiment was designed as per Animal Ethical Committee Guidelines. All the protocols and the
Chapter 3: Materials and Methods

Experiments were conducted in strict compliance with ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

3.1.6. Chemicals

Iron sulfate (CAS 7782-63-0), TQ (CAS 490-91-5), QCT (117-39-5), propidium iodide (P4170) (CAS 25535-16-4) and giemsa stain (CAS 51811-82-6) were procured from Sigma, USA. Other chemicals such as ethylene diamine tetraacetic acid (EDTA) disodium (054448), triton-X-100 (2020130), tris base (2044122), and 4- (2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES, 75277) were purchased from SRL, India. Potassium chloride (Merck-7447-40-7), sodium chloride (Merck-7647-14-5), sodium hydroxide (Merck-1310-73-2), methanol (Merck-67-56-1), glacial acetic acid (Merck-64-19-7), sodium bicarbonate (Merck-144-55-8), low melting point (LMP) agarose (Merck-9012-36-6), glycerol (Merck-56-81-5), colchicines (Merck-64-86-8), May-Grünwald's eosine-methylene blue solution modified (200-659-6) and xylene (1330-20-7) were purchased from Merck pvt ltd.

3.2. Chemical treatments

3.2.1. Route of administration

Intraperitoneal injection is the most commonly used method of administration largely for simplicity, and because, for many agents, it will tend to maximize chemical exposures to the bone marrow. However, this is not always the case, and other exposure routes can be equally or more effective. The route of administration of test compound in the present study was oral and intraperitoneal both.

3.2.2. Solvent or vehicle

Ferrous sulfate is water-soluble chemical so it was most desirable to dissolve the compound in water because exposure was oral for this chemical. The other chemicals like TQ and QCT were not soluble in water so were dissolved in organic solvents such as dimethyl sulfoxide (DMSO). The injection volume was kept as small as feasible i.e. accurate delivery was maintained. For chemicals in saline or water, volumes of 0.2-0.4 ml are recommended, but a maximum of 0.1 ml is necessary when DMSO is the solvent.
3.2.3. Dose selection

The doses of iron sulfate were selected on the basis of its effectiveness in inducing chromosomal aberrations and on the basis of available data (Benoni et al., 1993). Doses of TQ 6, 9, 12, 15, 18, 21 and 24 mg/kg b.w. were selected on the basis of available data that exhibited antimutagenic effects (Kaseb et al., 2007; Gali-Muhtasib et al., 2008). The doses of QCT (125, 250, 375, 500, 625, 750 and 875 mg/kg) were also determined on the basis of available data that exhibited antimutagenic effects (Da Silva et al., 2002; Leelayuwat et al., 2012).

3.2.4. Negative Control

A group of animals was included as control for the solvent (DMSO) used. The volume of solvent injected and the route of exposure were the same as that used for the groups treated with the test compounds.

3.2.5. Positive Control

Positive controls are included to establish the ability of analyzers to correctly ascertain aberrations, to determine the expected variations from test to test and animal to animal within an assay, and perhaps most importantly to establish the sensitivity of a particular test. Cyclophosphamide 25 mg/kg i.p. was used as a positive control in the present study.

3.2.6. Number of animals and administration of doses

The number of animals to be used for each group was large enough to lessen the effects of animal to animal variations in response, and also to enable the required number of cells to be obtained without analyzing a large number from any one animal. In the present study rats were divided in 23 groups of 6 animals each. The experiment was divided into three parts. The first part was done to determine the maximum genotoxic dose of iron sulfate among the doses of 50, 100 and 200 mgFe/kg on chromosomal aberration, micronucleus and Single cell gel electrophoresis (SCGE/Comet assay) in bone marrow and whole blood cells of rats. The rats were separated randomly into 5 groups of 6 animals each. The first group was used as control and administered with DMSO and second group was used as positive control and administered 25 mg/kg cyclophosphamide (i.p). Groups 3-5 were administered FeSO₄ at the doses of 50, 100 and 200 mg Fe/kg (p.o.). The second part was done to observe the optimum level of TQ
on chromosome aberrations, micronucleus and DNA damage induced by FeSO₄ 200 mgFe/kg. The rats were separated randomly into 9 groups of 6 animals each. Group 1 was used as control and administered with DMSO and Group 2 was administered FeSO₄ at the doses of 200 mg Fe/kg and group 3-9 were administered pre, simultaneous and post treatment of TQ at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg with the administration of FeSO₄ 200 mg Fe/kg.

The third part was done to observe the optimum level of QCT on chromosome aberrations, micronucleus and DNA damage induced by FeSO₄ 200 mg Fe/kg. The rats were separated randomly into 9 groups of 6 animals each. Group 1 was used as control and administered with DMSO and Group 2 was administered FeSO₄ at the dose of 200 mgFe/kg and groups 3-9 were administered pre, simultaneous and post treatment of QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg with the administration of FeSO₄ 200 mg Fe/kg.

3.3. Mammalian Bone Marrow Chromosomal Aberration Assay

3.3.1. Principles

The assay is based on the ability of a test agent to induce chromosome structural or numerical alterations that can be visualized microscopically. The target tissue for the chromosomal aberration assay is the bone marrow because it is a rapidly dividing, well vascularized tissue. A minimum of 5 rodents (mice, rats or Chinese hamsters) per sex per group are administered the test chemical, preferably only once, by an appropriate route of exposure, typically by gavage or intraperitoneal injection. Three doses are prepared so that they span a range from the maximum tolerable dose to a dose level that does not induce appreciable toxicity. The maximum tolerable dose should be identified in a preliminary test as that producing toxicity such that higher doses would be expected to lead to mortality, or the dose producing evidence of bone marrow cytotoxicity (i.e., >50% reduction of the mitotic index).

Following administration, sampling should usually be conducted twice. In order to accumulate metaphase cells, cell division is arrested by administration of a mitotic inhibitor, such as colchicine, 3-5 hours prior to sacrifice. Cells in their first metaphase after administration should be examined, so that any induced increase in the frequency of chromosomal aberrations can be related to the treatment. Therefore, half of the animals
should be sacrificed for the first sampling after a time period equivalent to 1.5x the normal cell cycle length (usually 12-18 hours for most species used). Because of the potential for some chemicals to induce mitotic delay, the remainder of the animals should be sacrificed 24 h after the first sampling time. Bone marrow cells are obtained immediately, exposed to hypotonic solution, fixed and stained. Using light microscopy, an observer should score a minimum of 1000 cells/animal for mitotic index and a minimum of 100 metaphase cells/animal for chromosomal aberrations. Cells scored for chromosomal aberrations should have a number of centromeres within the range 2n ± 2, because chromosomes can be lost during slide preparation. A test chemical that induces an increase in the frequency of structural aberrations, including chromosome-type and chromatid-type aberrations is considered to be clastogenic under the test conditions. A test chemical that induces an alteration in the frequency of aneuploid cells is considered to be aneugenic. There are two types of chromosomal aberrations as described below:

### 3.3.2. Sampling times and slide preparation

#### 3.3.2.1. Sampling time

Rats were sacrificed after 24 h of each treatment by using ether as anesthesia. All procedures were carried out according to the international practices for animal use and care. In order to accumulate metaphase cells, and provide more readily analyzable chromosomes a mitotic inhibitor (Colchicine- 2 mg/kg body weight) was injected i.p. usually 2 h before killing the animals.

#### 3.3.2.2. Slide preparation, Sacrifice and harvest

Femurs were quickly removed, muscle was cleaned from the bone and both femurs were placed on the edge of a prenumbered plastic centrifuge tube which corresponds to the animal number. The cells in bone marrow were flushed with a hypodermic syringe fitted with a 22-g needle by flushing 0.075 M KCl 2-3 times into the marrow cavity of femur until after careful observation no bone marrow remains attached to bone. After a few seconds to allow fragments to settle, the suspension was decanted to the other corresponding tube. The tubes were centrifuged for about 10 min at 1000 rpm. Supernatant was removed by gentle aspiration unit a small volume remains above the pellet. The pellet was resuspended in the remaining volume.
3.3.2.3. Hypotonic treatment

0.075 M KCl (prewarmed to 37°C) was added drop wise with agitation to approximately 5 ml. it was incubated for 20 min in 37°C water bath and centrifuged at 1,000 rpm for 10 min. The supernatant was removed. The pellet was resuspended in the remaining volume.

3.3.2.4. Fixation

0.5 ml fixative (3:1 absolute methanol: glacial acetic acid made freshly immediately before using) was added drop wise using a Pasteur pipette while continuously shaking the pellet so as to avoid formation of clots. It was allowed to stand at room temperature for 15-20 min. In order to ensure proper fixation, the cells were kept suspended in the fixative at 4°C for a minimum period of 1-h but preferably overnight. The contents, then, were again centrifuged at 1000 rpm for 10 minutes and the same two or three changes with fresh fixative were given before preparing the slide.

3.3.2.5. Slide preparation

After giving final washing in the fixative, the cells were resuspended in 0.2 ml of fresh fixative. Slides were prepared by flame drying technique. Slides were washed clean and immediately coded and kept at 4°C in distilled water. About 3-4 drops of cell suspension were dropped per slide with the help of Pasteur's pipette over the chilled, tilted slides maintaining a stretched hand distance between the slides and the pipette to get better spreading, and a brief exposure to the flame was given to dry the slides. Three drops/slide was generally enough to get a satisfactory cell count. The slides were kept in a slide box under dust free conditions for further use. After one-hour slides were stained in 5% Giemsa stain in phosphate buffer (pH 6.8).

3.3.2.6. Scoring and Photography

At least 100 well-spread metaphases have to be scored per animal of each treatment and control. The experimental unit is the cell, and therefore the numbers of cells with structural chromosome aberrations have to be evaluated. Detectable and finely spread metaphase chromosome spots were analyzed under 100X oil immersion, position of the desired spot was marked by reading scales over the microscope. Photographs were taken with an automatic digital camera attached in a Nikon microscope 80i.
3.4. Micronucleus Assay

3.4.1. Principles

The micronucleus assay detects chromosome damage and whole chromosome loss in polychromatic erythrocytes, and eventually in normochromatic erythrocytes in peripheral blood as the red cells mature. A micronucleus is a small structure (1/5 to 1/20 the size of the nucleus) containing nuclear DNA that has arisen from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei at anaphase of mitosis. Micronuclei can be found in cells of any tissue, but only form in dividing cells. There are four generally accepted mechanisms through which micronuclei can form: a) the mitotic loss of acentric chromosome fragments (forming structural aberrations), b) mechanical consequences of chromosomal breakage and exchange, such as from lagging chromosomes, an inactive centromere or tangled chromosomes (forming structural aberrations), c) mitotic loss of whole chromosomes (forming numerical aberrations) and, d) apoptosis. However, nuclear fragments resulting from apoptosis are usually easy to identify because they are much more numerous or pyknotic than those induced by clastogenic or aneugenic mechanisms. Structural aberrations are believed to result from direct or indirect interaction of the test chemical with DNA, while numerical aberrations are often a result of interference with the mitotic apparatus preventing normal nuclear division.

Bone marrow is the major haematopoietic tissue in the adult rodent. Administration of a chemical during proliferation of haematopoietic cells may cause chromosome damage or inhibition of the mitotic apparatus. These chromosome fragments or whole chromosomes may lag behind during cell division and form micronuclei. The erythrocyte is particularly well suited to analysis for micronuclei because during maturation of the erythroblast to the polychromatic erythrocyte (a period of about 6 hours following the final mitosis), the nucleus is extruded, making detection of micronuclei easier. In addition, the polychromatic erythrocyte (PCE) still contains RNA, and so it stains blue-grey with Giemsa or reddish with acridine orange. This allows differentiation from mature, haemoglobin-containing erythrocytes (NCE), which stain orange with Giemsa or are unstained by acridine orange, and facilitates identification of the cells where micronuclei induced by the test substance may be present. Sampling of PCEs from the bone marrow or peripheral blood prior to their differentiation to mature erythrocytes is critical; once a
PCE has matured, associating the presence of micronuclei in these cells with acute chemical exposure is not possible. Mature erythrocytes persist in peripheral circulation for about 1 month.

The micronucleus assay is conducted using the bone marrow or peripheral blood of rodents, typically mice, as the target tissue; the peripheral blood of species other than the mouse can be used if there is evidence that demonstrates micronucleated erythrocytes are not rapidly removed by the spleen. The test chemical is administered to a minimum of five animals of each sex per group orally by gavage or by intraperitoneal injection. Three doses are prepared so that the dose range spans a range from the maximum tolerable dose to a dose level that does not induce appreciable toxicity. One of two treatment schedules is recommended: the test chemical is administered once and one group is sacrificed at 24 h and another at 48 hours after treatment (for bone marrow), or at 36 and 72 hours after treatment (for peripheral blood); if multiple treatments are used, a single sample can be taken between 18-24 h after the last administration for bone marrow or between 36-48 hours for peripheral blood. A delay between chemical administration and sampling of PCEs is necessary to allow sufficient time for the number of micronucleated PCEs to rise to a peak. This corresponds to the time necessary for absorption and metabolism of the chemical, the completion of the erythroblast cell cycle, including any test chemical-induced cell-cycle delay, and for extrusion of the erythroblast nucleus. Because the incidence of micronucleated PCEs is rare in untreated animals, to allow for appropriate statistical power, at least 2000 PCEs per animal should be scored for the incidence of micronuclei. The proportion of PCE among total erythrocytes should also be determined from at least 200 total erythrocytes for bone marrow samples or from at least 1000 for peripheral blood samples. Data indicating the test chemical depressed the PCE: total erythrocyte (TE) ratio are suggestive of cytotoxicity and provide evidence that the test chemical reached the bone marrow. However, significant clinical symptoms of toxicity or plasma test chemical concentrations may also be used as a measure of exposure, since the bone marrow is a relatively well-perfused tissue and the plasma test substance concentration should approximate the level of bone marrow exposure. However, if the test chemical is severely cytotoxic, finding and scoring 2000 PCEs at higher concentrations may be difficult; in these circumstances it is particularly important to have multiple dose groups. Because micronuclei are relatively rare, manual enumeration
by light microscopy is time consuming. For that reason, newer flow cytometric or image analysis methods have been adapted for the rapid processing of slides. These methods have yet to undergo a thorough evaluation, but they offer tremendous potential for improving the sensitivity and the efficiency of the assay. Any test chemical that induces an increase in the frequency of micronucleated PCE is concluded to have induced chromosomal aberrations in vivo, but further mechanistic information useful to distinguish micronuclei induced by clastogenic or aneugenic chemicals can be obtained. Micronuclei of aneugenic origin will contain centromeres, the presence of which can be verified using one of two molecular cytogenetic methods: immunofluorescent CREST-staining or fluorescence in situ hybridization (FISH) with pancentromeric DNA probes. This information is useful in risk assessment because aneugens may exhibit threshold dose-responses that are not typical of those chemicals that interact directly with DNA.

3.4.2. Sampling times and slide preparation

3.4.2.1. Sampling time

Rats were sacrificed after 24 h of each treatment by using ether as anesthesia. All procedures were carried out according to the international practices for animal use and care.

3.4.2.2. Extraction of bone marrow from animals

This assay was performed according to the method described by Schmid (1975). Before sacrifice the animals, a 5-ml centrifuge tube is filled with fetal calf serum for each individual. This admittedly expensive fluid has proved superior to all other rinsing solutions or cheaper sera tried so far. With substitutes the cells were often damaged or the erythrocytes were partially agglutinated resulting in preparations that were unusable or consumed too much time for analysis. From a single rat one can easily obtain enough bone marrow cells for several slides. From the freshly killed animal both femora are removed in toto. The bones are then freed from muscle by the use of gauze and figureis. The proximal end of the femur is carefully shortened with scissors until a small opening to the marrow canal becomes visible. With the needle of appropriate size mounted, about 0.2 ml serum is pulled from the tube into a disposable plastic syringe. Then the needle is inserted a few mm into the proximal part of the marrow canal which is still closed at the distal end. Next, the femur is submerged completely in the serum and squeezed against
the tube to prevent the bone from slipping off the needle. Subsequently, the marrow is aspirated; should the needle have become obstructed, the serum in the syringe is first pressed out. After several gentle aspirations and flushings, the process is repeated from the distal end of the femur. The bone marrow cells should get into the serum as a fine suspension and not in the form of gross particles.

3.4.2.3. Preparation of the smears

The tube is centrifuged at 1000 rpm for 5 min. The supernatant is removed with a Pasteur pipette. If the sediment is large, half a drop of serum is left; if it is minute, all the supernatant is drawn off. The cells in the sediment are carefully mixed by aspiration into the capillary part of a fresh, siliconized Pasteur pipette. A small drop of the viscous suspension is put on the end of a slide and spread by pulling the material behind a polished cover glass held at an angle of 45 degrees. The size of the droplet is chosen so that all material is used up at a distance of 2-3 cm. The preparations are then air dried.

3.4.2.4. Staining

The best results are obtained if staining takes place the day following preparation. If staining is done immediately, the slides must be flamed shortly. Staining is carried out in ordinary vertical staining jars according to the following procedure: staining for 3 min in undiluted May-Gruenwald solution; staining for 2 more min in May-Gruenwald diluted with distilled water 1:1; staining for 10 min in Giemsa diluted with distilled water 1:6; rinsed in distilled water; blotted dried with filter paper; cleaned back side of slide with methanol; cleared in xylene for 5 min, and mount with cover glass.

3.4.2.5. Analyzing the slides

First the slides are screened, at medium magnification, for regions of suitable technical quality, where the cells are well spread, undamaged and perfectly stained. Such regions are normally located in a zone close to the end of the smear. A perfect morphology of the nucleated cells serves as criterion for good quality, even though the nucleated cells are not evaluated in the test. The erythrocytes must be well spread, neither globular nor having slurred contours. Their staining has to be vigorous, red in mature erythrocytes (anulocytes) and with a strong bluish tint in the immature forms (polychromatic erythrocytes). For each animal, three slides were prepared and the best slide was selected.
for scoring. The incidences of micronuclei (MN), observed in 2000 PCE/rat were calculated to find the clastogenic property of the test chemicals.

3.5. Single cell gel electrophoresis (SCGE/Comet assay)

3.5.1. Principle of Comet assay

In the Comet assay, the cells are embedded in a thin agarose gel on a microscope slide. The cells are then lysed to remove all cellular proteins and the DNA is allowed to unwind under alkaline/neutral conditions. Following unwinding the DNA is electrophoresed and stained with a fluorescent dye. During electrophoresis, broken DNA fragments (damaged DNA) or relaxed chromatin migrates away from the nucleus. The extent of DNA liberated from the head of the comet was directly proportional to the DNA damage. There are many possible explanations on how comet assay works. Many evidence points to the fact that during the lysis step, the membranes, cytoplasm and nucleoplasm are removed leaving a halo of DNA embedded in agarose. DNA damage induces relaxation in the super-coiled DNA coils, which are then pulled to one side by electrophoresis. According to this theory, a single strand break should uncoil and relax the DNA, which can then be picked up on the Comet assay. There are also evidences suggesting that DNA damage induces small fragments of DNA which are then separated on electrophoresis. Comet assay essentially measures the degree of relaxation as well as fragmentation of DNA within the cell. It is therefore necessary to convert DNA damage into strand breaks that relax the super-coiled DNA or produce DNA fragments before detected on Comet assay. The simplest types of DNA damage detected by Comet assay are the Double Strand Breaks (DSBs). DSBs within the DNA results in DNA fragments and can be detected by merely subjecting them to electrophoretic mobility at the neutral pH. Single Strand Breaks (SSBs) does not produce DNA fragments unless the two strands of the DNA are separated/denatured. This is accomplished by unwinding the DNA at pH 12.1. It is also possible that single strand breaks can relax the DNA and hence can also be detected on Comet assay at a neutral pH. Other types of DNA damage broadly termed as alkali labile sites (ALS) are expressed when the DNA is treated with alkali at pH greater than pH13. Furthermore, breaks can be introduced at the sites of DNA base modifications by treating the DNA with lesion-specific glycosylases/endonucleases and the fragments thus produced can also be detected by Comet assay. Therefore, by controlling the conditions that produces nicks at the sites of specific DNA
lesions, Comet assay can be used to detect various classes of DNA damage. While breaks increase DNA migration, DNA binding and Crosslinks (DNA-DNA or DNA-Protein Interactions) can retard DNA migrations, and can also be detected on Comet assay.

**Definition of some important comet parameters**

**Tail Length:** Tail Length is the distance of DNA migration from the body of the nuclear core and it is used to evaluate the extent of DNA damage.

**Olive Tail Moment:** Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail). Olive Tail moment = \((\text{Tail. mean - Head. mean}) \times \frac{\text{Tail}\%\text{DNA}}{100}\).

**Extent Tail Moment** = \((\text{Tail Length} \times \text{Tail}\%\text{DNA}) / 100\).

**Head \% DNA** = \([\frac{\text{Head.Opt-Inten}}{(\text{Head.Opt-Inten} + \text{Tail.Opt-Inten})}] \times 100\).

**Tail \% DNA** = 100 – Head \% DNA.

**3.5.2. Sampling times and slide preparation**

**3.5.2.1. Sampling time**

Rats were sacrificed 24 h after each treatment by using ether as anesthesia. All procedures were carried out according to the international practices for animal use and care.

**3.5.2.2. Blood collection**

Blood samples were collected by cardiac puncture using a 21-gauge needle and syringe, and immediately transferred into tubes containing sterile EDTA solution (to a final concentration of 1.6 mg EDTA/ml of blood). Blood was processed immediately for comet assay.

**3.5.2.3. Slide preparation**

This assay was performed in dark according to the method described by Buschini et al. (2002) with slight modifications. 0.8% low melting point (LMP) agarose was prepared in
saline and was maintained at 39°C to prevent solidification. Subsequently, 20µl of whole blood treated with iron sulfate alone and/or iron sulfate along with different concentrations of TQ and QCT were gently mixed with 250µl of 0.8% LMP agarose. The resulting suspension was layered onto the frosted side of fully frosted slides. The slides were placed on ice for approximately 5 min to allow the agarose to solidify.

3.5.2.4. Lysis

Once prepared, slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA with fresh 1% Triton-X-100 and 10% DMSO) for 1 h to eliminate non-nuclear components prior to the alkali unwinding step.

3.5.2.5. Unwinding and electrophoresis

The slides were further immersed in alkaline buffer (300 mM NaOH, 1 mM EDTA, pH = 13) for 20 min to allow the DNA to unwind and to convert alkali labile sites to single strand breaks. Electrophoresis was conducted for 30 min at 15 V and 200 mA (at a rate of 0.6 V/cm) using a compact power supply.

3.5.2.6. Neutralization and dehydration of slides

After completion of electrophoresis, the slides were gently washed with 0.4 M Tris (pH = 7.5) to remove alkali and detergents for at least 10 minutes.

3.5.2.7. DNA staining, comet visualization and analysis

The slides were placed in a humid chamber until staining to prevent the gel from drying. The cells were stained with propidium iodide (PI – 20 µg/ml) and were observed under a fluorescent microscope. Images of the cells were obtained using a digital camera. Approximately 60–80 images per slide were captured from different imaging fields and were analyzed with the appropriate software. For each image, the two SCGE parameters, including the olive tail moment (OTM) and tail moment (TM) were analyzed. Olive tail moment = (tail mean – head mean) × tail%DNA/100; tail moment is = tail length × tail%DNA (tail intensity)/100.