

DNA DAMAGE AS APOPTOTIC FACTOR IN *HUMAN* MESENCHYMAL STEM CELLS DURING *TOXOPLASMA GONDII* INFECTION

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SUMMARY

Toxoplasma gondii is a parasitic worm that infects all warm-blooded creatures on the planet. When it infects a cell, it manipulates the host's DNA damage response, which is still unknown. The study's goals were to I analyze DNA damage in *T. gondii*-infected cells in vitro, and (ii) figure out what causes DNA damage in *T. gondii*-infected cells effects of *T. gondii*-induced apoptosis and DNA damage in human umbilical cord mesenchymal stem cells were investigated using DNA fragmentation analysis. The extent of DNA damage was measured using the comet test to determine the level of DNA damage. This study included three groups: control, nutritionally infected, and in vitro infected. Infected hMSCs showed a considerable increase in DNA damage in the comet tail when compared to the control group. In vitro infected samples had substantially more severely damaged spots than naturally infected samples. *T. gondii* infection caused DNA damage in infected MSCs.

Keywords: DNA, Fragmentation, MSCs, *T.gondii*, Apoptosis, infected cell .

I. INTRODUCTION

Toxoplasmosis is a wide spread disease caused by the obligatory intracellular protozoan parasite of Class Apicomplexa, *T. gondii*. It is ubiquitously distributed infecting a wide range of warm blooded hosts, and over a billion individuals constituting more than 20% of the human population worldwide [1].

Ingestion of undercooked meat containing parasite cysts and ingestion of oocysts transported into the environment by cats' feces are the two main routes of human infection [2]. Tachyzoites multiply quickly during the acute stage of toxoplasmosis Infected people produce a powerful immune response that effectively inhibits the parasite's growth within 2-3 weeks. Some tachyzoites mature into latent bradyzoites, which grow at a considerably slower rate than tachyzoites and eventually become encased in a dense matrix with a thick wall, forming a tissue cyst [3-5].

Tissue cysts in the brain, heart, and skeletal muscles of people who have previously been infected with *T. gondii* can last for years [6]. *Toxoplasma gondii* is a protozoan parasite that infects practically all warm-blooded animals, including humans, all over the planet [7]. It facilitates the infected cell's survival and proliferation by modulating some biological processes such as autophagy and apoptosis [8]. DNA damage was also found in *T. gondii*-infected mice's retina and peripheral blood cells [9]. It is, however, still unknown how *T. gondii* damages the host's DNA and how the host responds to the damage. DNA fragmentation is a typical biochemical downstream signature of apoptosis in mammals [10] and is a key marker for protozoan apoptosis-like cell death [11, 33-44].

The assay of single cell electrophoresis, commonly known as the comet assay, is a technique for measuring and assessing DNA breakage in mammalian cells that is quick, easy, visible, and sensitive. The goal of this study is to see if toxoplasmosis causes DNA damage and how much damage it causes in human mesenchymal stem cells.

II. MATERIAL AND METHODS

Study Sample

This study included samples from the infected with *T gondii* and uninfected umbilical cord and placenta by 50 healthy samples and 20 infected samples. All samples were examined by detection IgG and IgM *Toxoplasma* antibodies to confirm whether they were infected with the parasite. Infected and uninfected samples were collected from maternity and caesarean sections at the Maternity and Children Hospital in Al-Ramadi city in agreement with obstetricians and gynecologists in the hospital and outpatient clinics, after obtaining official approvals. The umbilical cord and placentas samples are stored in sterile containers containing antibiotics with about one liter of normal saline and kept at (20C°) for a period of one to 12 hours as a maximum.

WJ of umbilical cord Isolation

After obtaining the entire umbilical cord, the umbilical cords are then cut into equal pieces of size and length about 2-5 cm. All the pieces are incised longitudinally to get rid of the blood vessels inside each piece. The pieces are placed inside sterile tubes containing the enzymatic solution consisting of Collagenase and Hyaluronidase in order to break down the tissues and free the cells to be culture later. Tubes containing Wharton's jelly and cells are incubated for 12 to 24 hours. The tubes containing samples are centrifuged (1000 rpm for 5 min) and the precipitate is kept until cultured.

Culture and passed MSCs

Culture of MSCs in DMEM (low-glucose Dulbecco's modified Eagle medium) which contend 10 % FBS and 2 ml per L l-glutamine, and antibiotics . after 48 h cells were washed with PBS and added fresh medium. culture medium was changed (passed) after three day of culture after obtaining a concentration of 9×10^6 cells twice a week by changing a half medium. cells are washed 3 times with PBS solution, trypsin is added to suspend adherent cells and cells are incubated at 37 °C for 5 minutes, then 5 ml of media is added to obtain a single cell suspension. Cell viability tested by using trypan blue dye which permits distinguishing among wholesome cells with uncompromised membrane integrity (unstained) and useless ones (stained blue).

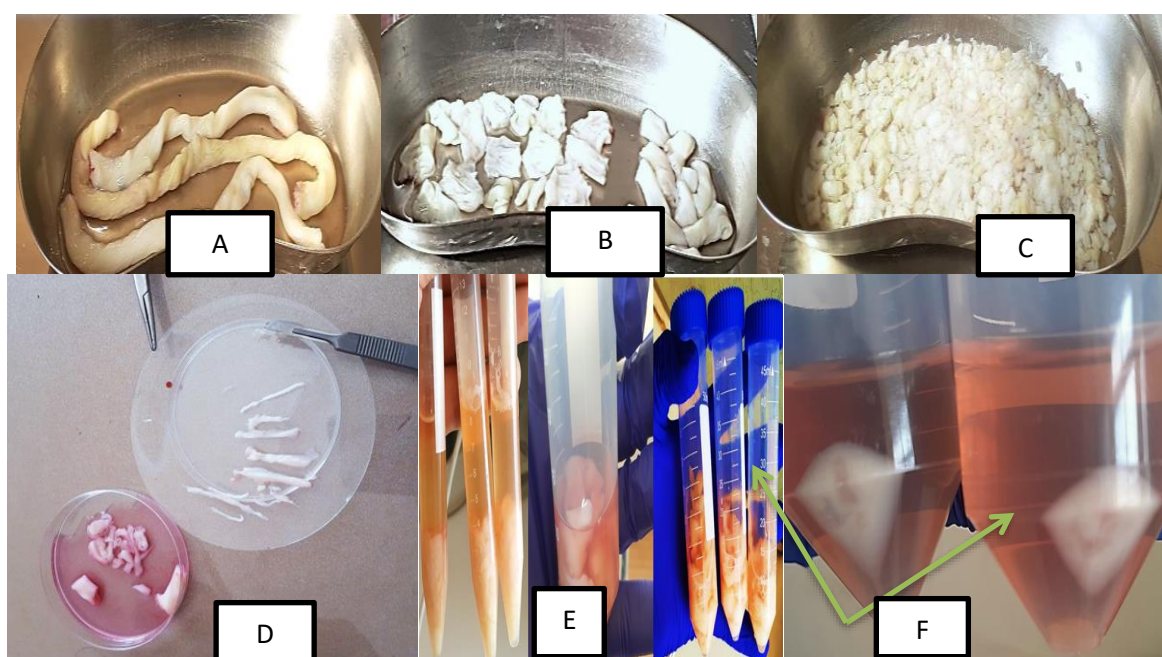


Fig 1: Steps to isolate MSCs , A / umbilical cord of Human. B,C/ Cut of Human umbilical cord into long and small pieces., D/ Removed of Blood vessels from a piece of umbilical cord tissue . E/ Umbilical cord tissue in enzyme solution . F/ Pellet after collagenase digestion.

Phenotypic analysis

hUC-MSCs were extracted twice with PBS after passage 3 (P3) washing. The cells were then characterized utilizing an Immunocytochemistry study of MSCs to look at the expression of the markers CD90, CD106, and CD34. All materials and operating techniques were prepared according to the company's instructions for cd markers supplied by (US- Biological-USA).

Cell culture and *T. gondii*

With 0.25 % trypsin, the cells were removed from the cell culture flask. Every 2-3 days, the passage operation was repeated. Tachyzoites of *T. gondii* were kept alive in MSC cells. The MSCs cells media was changed to DMEM medium with 2% fetal bovine serum before infection with *T. gondii* tachyzoites for 12 hours. 4 hours after infection, the *T. gondii* tachyzoites were introduced to the culture medium, which was then replaced with new fresh DMEM medium (containing 2% fetal bovine serum). Centrifugation was used to harvest MSCs cells and tachyzoites [12].

The assay of Immunofluorescence

T. gondii infection rates were assessed by incubating hUC-MSC monolayers on glass coverslips for 4, 8, 12, and 24 hours. The cells were fixed with 4 percent paraformaldehyde and permeabilized with 0.1 percent Triton X-100 in PBS (PBST) for 10 minutes after treatment. To label F-actin, the coverslips were rinsed with PBST and stained with Texas Red-X phalloidin (ThermoFisher - USA). Finally, the coverslips were cleaned and placed onto microscope slides with DAPI-containing mounting solution (Sigma-Aldrich Germany), and the cells were examined using fluorescence microscopy. The number of parasite-infected cells was counted and expressed as a proportion of total cells

Cell viability assay

After infecting hUC-MSCs with *T. gondii* for 4, 8, 12, or 24 hours, the viability of the cells was determined using the Cell Proliferation Reagent WST-1 Cell Proliferation Assay kit (Roche - Germany), according to the manufacturer's instructions.

Assessment of DNA damage

The Comet assay from Cell Biolabs is a single-cell gel electrophoresis (SCGE) assay for assessing cellular DNA damage. Before being applied to the Comet Slide, individual cells are combined with molten agarose. The DNA in these implanted cells is then relaxed and denatured using a lysis buffer and alkaline solution. Finally, the samples are separated into intact and damaged DNA fragments by electrophoresis in a horizontal chamber. The samples are dried, dyed with a DNA dye, and observed using epifluorescence microscopy after electrophoresis. Damaged DNA (including cleavage and strand breaks) will move further than undamaged DNA under these conditions, forming a "comet tail" structure (see Figure 1).

Statistical Analysis:

To detect the effect of different components in research parameters, the Statistical Analysis System- SAS (2012) application was utilized. In this study, the least significant difference –LSD test (ANOVA) was utilized to make a significant comparison between means.

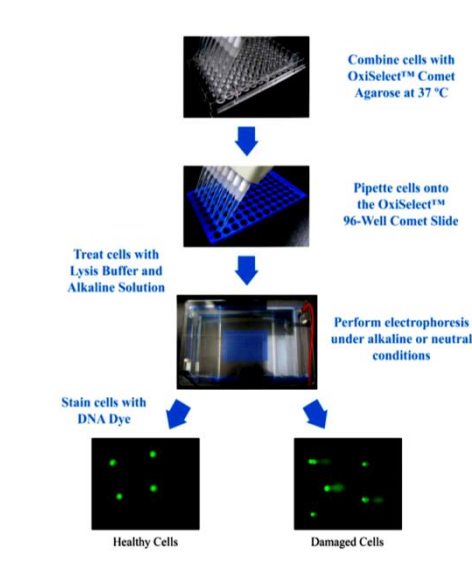


Fig 1. Comet Assay Principle

III. RESULTS

3.1. Morphology of mesenchymal stem cells

Morphological identification of human WJ MSCs using inverted microscope: After 24 hours from the primary culture (passage 0 = P0) of human umbilical cord WJ MSCs, the cultured cells from samples The cultured Cells appeared in a weak, undifferentiated and unclear-looking form. (fig .2A)

After three days, the cultured cells appeared crowded and suspended. , Cells appear more clearly and the nuclei are more clear . (fig 2B)

Three days after the cell transplant, the cells were passed on for the first time . They came in a variety of sizes and shapes. The majority of the cells seem spherical, however some cells appear rounded, refractile, and nonadherent as human umbilical cord WJ-MSCs from the past culture (passage 1) (hematopoietic stem cells). MSCs were grouped in the shape of tiny colonies, whereas hematopoietic stem cells were not connected to the culture flasks. .(fig.2C)

The adherent cells reached 80-90%confluency after Seven days from the primary culture, and appear triangular, star shaped and spindle shaped. Some of the cells exhibited two vesicular nuclei . (fig.2D)

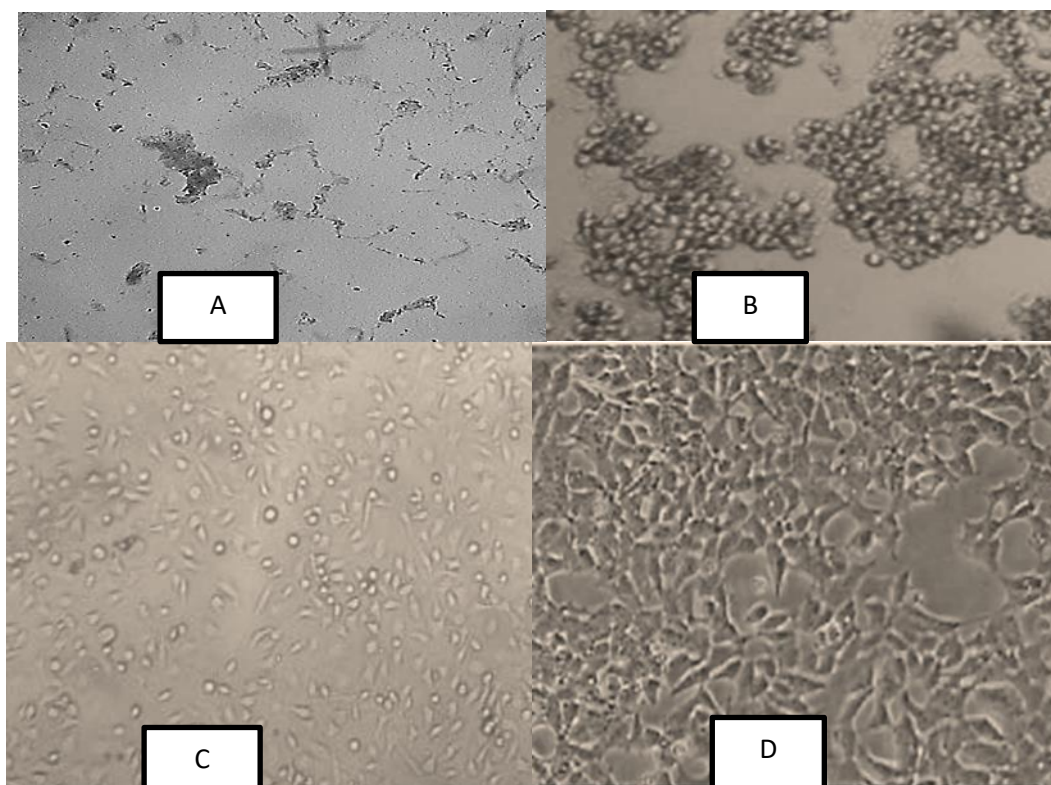


FIG.2 A/ Phase contrast micrographs of human umbilical cord WJ-MSCs 24 hours from the primary culture (passage 0 = P0) of human umbilical cord WJ MSCs, the cultured cells from samples The cultured Cells appeared in a weak, undifferentiated and unclear-looking form. B/ Phase contrast micrographs of human umbilical cord WJ-MSCs After three days, the cultured cells appeared crowded and suspended. , Cells appear more clearly and the nuclei are more clear . C/ Phase contrast micrographs of human umbilical cord WJ-MSCs on 3th day from passed culture (passage 1 = P1) of human umbilical cord mesenchymal stem cells, some cells appear rounded, refractile and nonadherent (hematopoietic stem cells).(P1 × 200). D/ Seven days from the primary culture, The adherent cells reached 80-90% confluency and appear triangular, star shaped and spindle shaped. Some of the cells exhibited two vesicular nuclei (Passeg 3) .

Immunophenotypic characterization of WJ-MSCs

Immunocytochemistry staining analysis revealed the morphological homogeneity of the monolayer cultures at the third passage on falcon. We should have used many MSC markers for the standard method, but due to a lack of these markers, we only used three confirmative markers. CD-surface indicators immunocytochemistry:

immunological profiling, cell passage 3, positive expression for mesenchymal stem markers CD44 and CD106. On the other hand, CD34 marker showed negative expression alone and when it is combined with CD106 marker. (Fig 2.A,B).

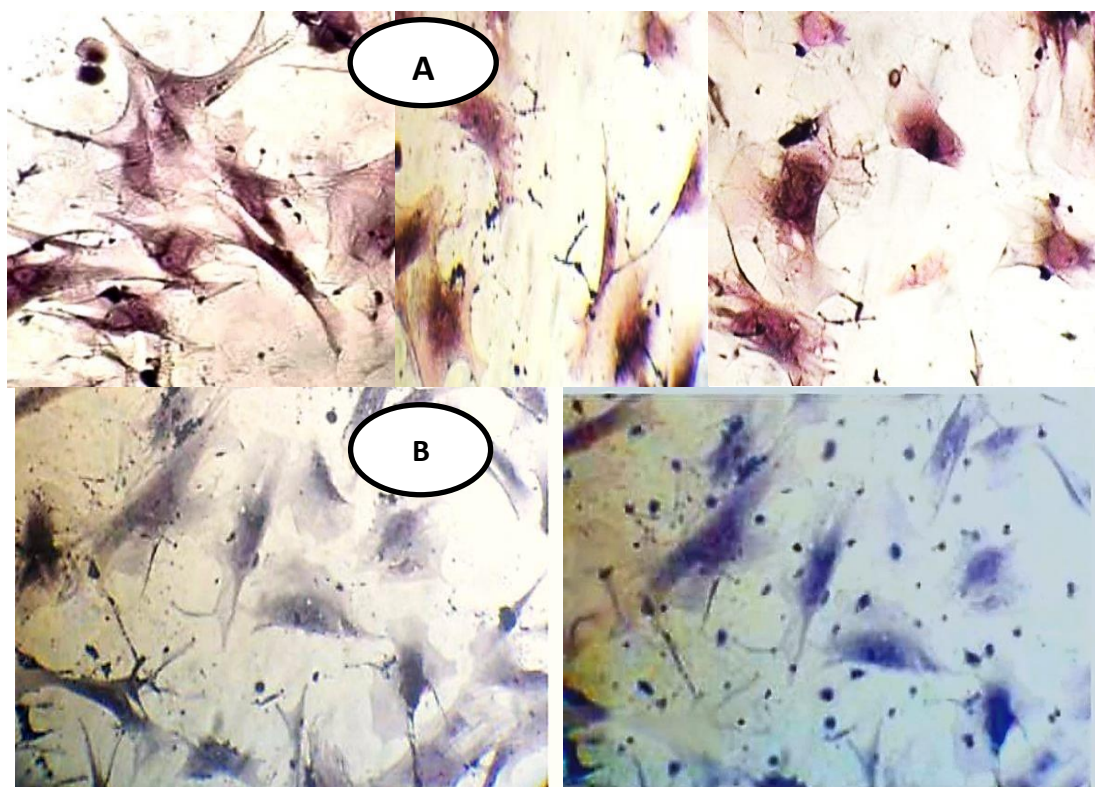


Fig.3 By using a light microscope, MSC immunocytochemical investigation of mesenchymal stem cells was shown. **(A)** : WJ-MSCs The majority of adhering MSCs responded positively to the CD44 and CD106 markers, which were stained with a brown DAB stain and hematoxylin stain (20X). **(B)**: WJ-MSCs The majority of adhering MSCs had a negative reaction to the CD 34 marker and were stained with brown DAB stain and hematoxylin stain(20X).

Cell viability and cell Proliferation

At the cellular level, cell vitality was examined after each cell pass using trypan blue dye, a routine procedure after each pass to confirm cell vitality and the success of the cellular transplant. The results of the count using the dye showed increased the proportion of living cells in samples isolated from uninfected umbilical cords by 45.3 (87.7%) On the contrary, the proportion of dead cells in the samples was 6.4 (12.3%). On the contrary, isolated cell samples from infected umbilical cords showed a decrease in the proportion of living cells to 7.2 (22 percent). With the percentage of dead cells rising to 25.6 (78%) .

infecting hUC-MSCs with *T. gondii* for 2 hours, 4 hours, 8 hours, and 12 hours, we examined cell viability using the Cell Proliferation Reagent WST-1 for 1/2, 1, 2, and 4 hours. In comparison to control cells that had not been infected, and After infection, the cells were inspected to validate the parasite's dominance over the cells and to confirm the events of the infection in vitro by labeling F-actin with Texas Red-X phalloidin. The viability of cells infected with *T. gondii* for 2 and 4 hours was considerably reduced by 21% and 30%, respectively. (fig 4) We looked at the microscopic morphology of *T. gondii*-infected hUC-MSCs and found that they had an extended shape relative to untreated control cells (Fig. 5 A,B,C,D,E). *T. gondii* infection of hUC-MSCs resulted in considerable cell death in a time-dependent manner, according to our findings

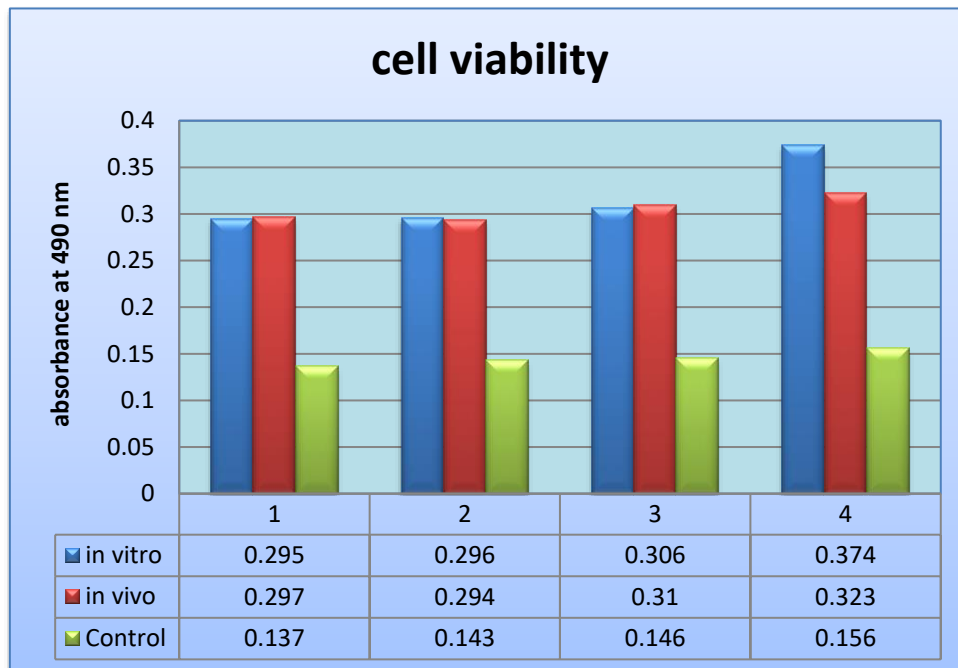


Fig 4 cell viability was measured by Cell Proliferation Reagent WST-1

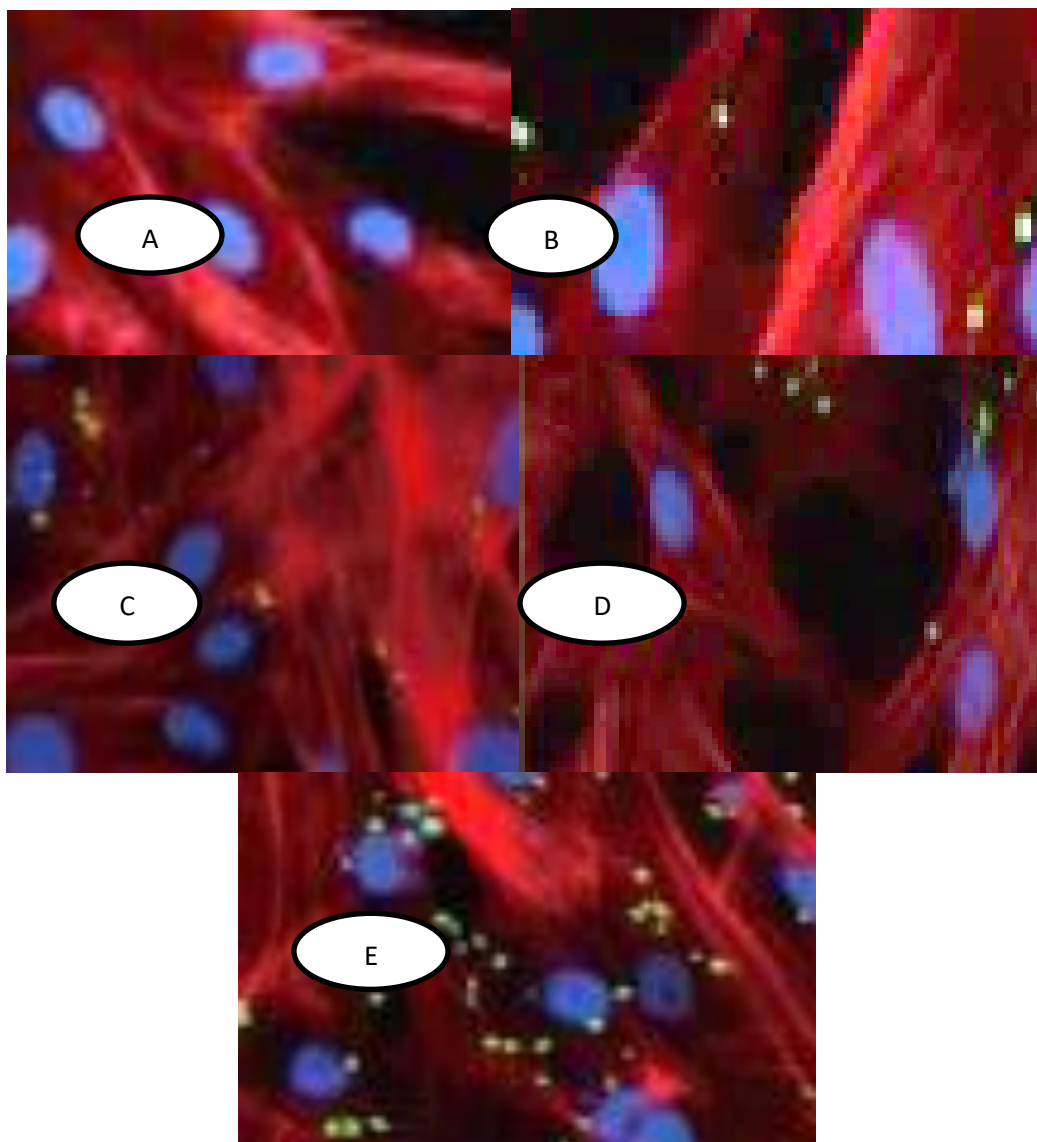


Fig 5. *T. gondii* was inoculated into hUC-MSCs for the time periods indicated. To label F-actin (red), cells were fixed and stained with Texas Red-X phalloidin, and nuclei with DAPI (blue). B,D,D,E infected cells after infection, A/control, B,D,D,E infected cells after infection

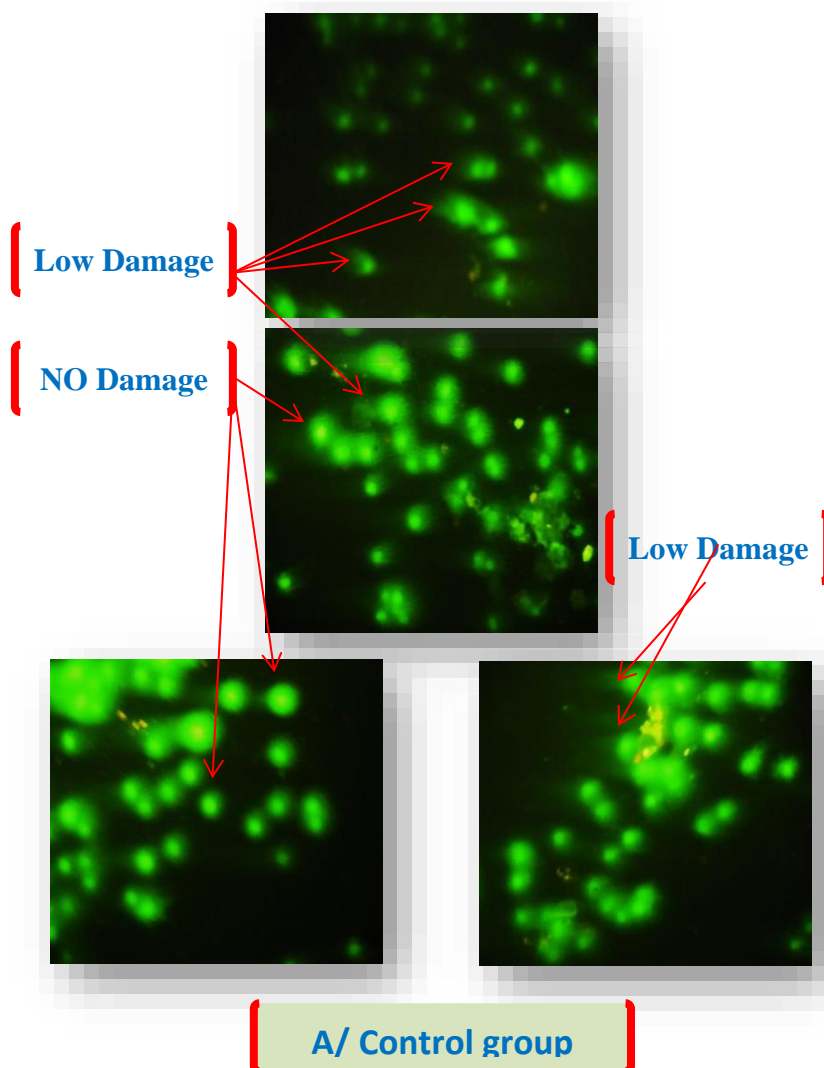
Infection with *T. gondii* can cause DNA damage in mesenchymal stem cells

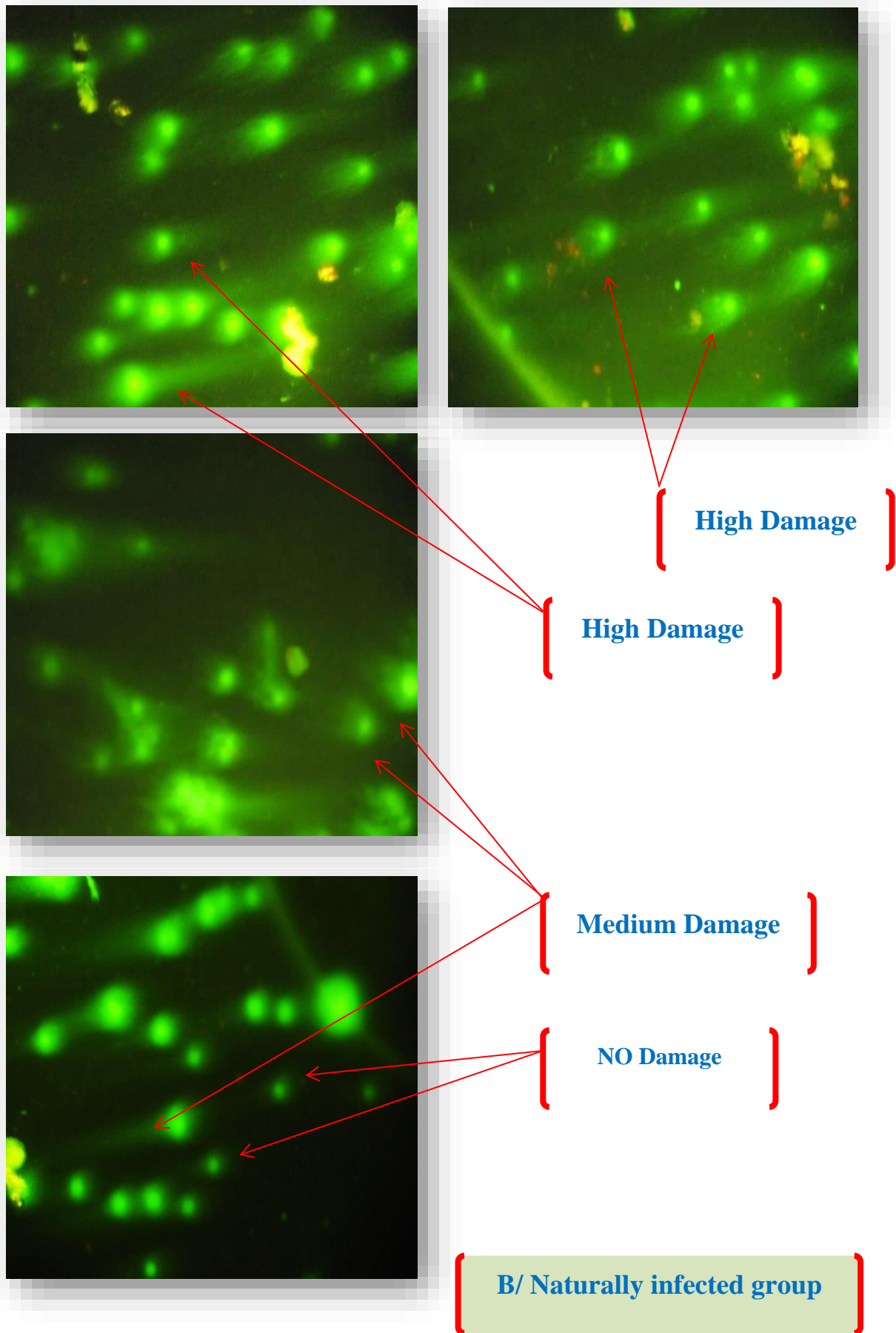
The damage in the DNA was evaluated using a score software analysis image comet, as the important criteria used to know the percentage of damage in the DNA was the severity of the breaks, so given the ratio of high damage, medium damage, low damage, no damage. A speedy, simple, visible, and reliable biochemical technique called single-cell gel electrophoresis test (comet assay) was utilized to detect numerous proteins. [13] .

In eukaryotic cells, this approach can detect single and double strand breaks as well as incomplete repair sites. In the comet experiment, quantitative examination for DNA damage provided a number of metrics, including tailed nuclei, tail length, percentage of DNA in the tail, and tail moment. [14] .

DNA damage such as strand breakage and DNA-protein crosslinks were detected using the Comet test. Cross-links can help to stabilize chromosomal DNA and prevent it from migrating. [15].

It was clear from the results of our current study in Figure (4.6), that the highest percentage of DNA breakdowns was in the group of Naturally infected and in vitro infected samples . It was clear from the pictures and (fig .7) .





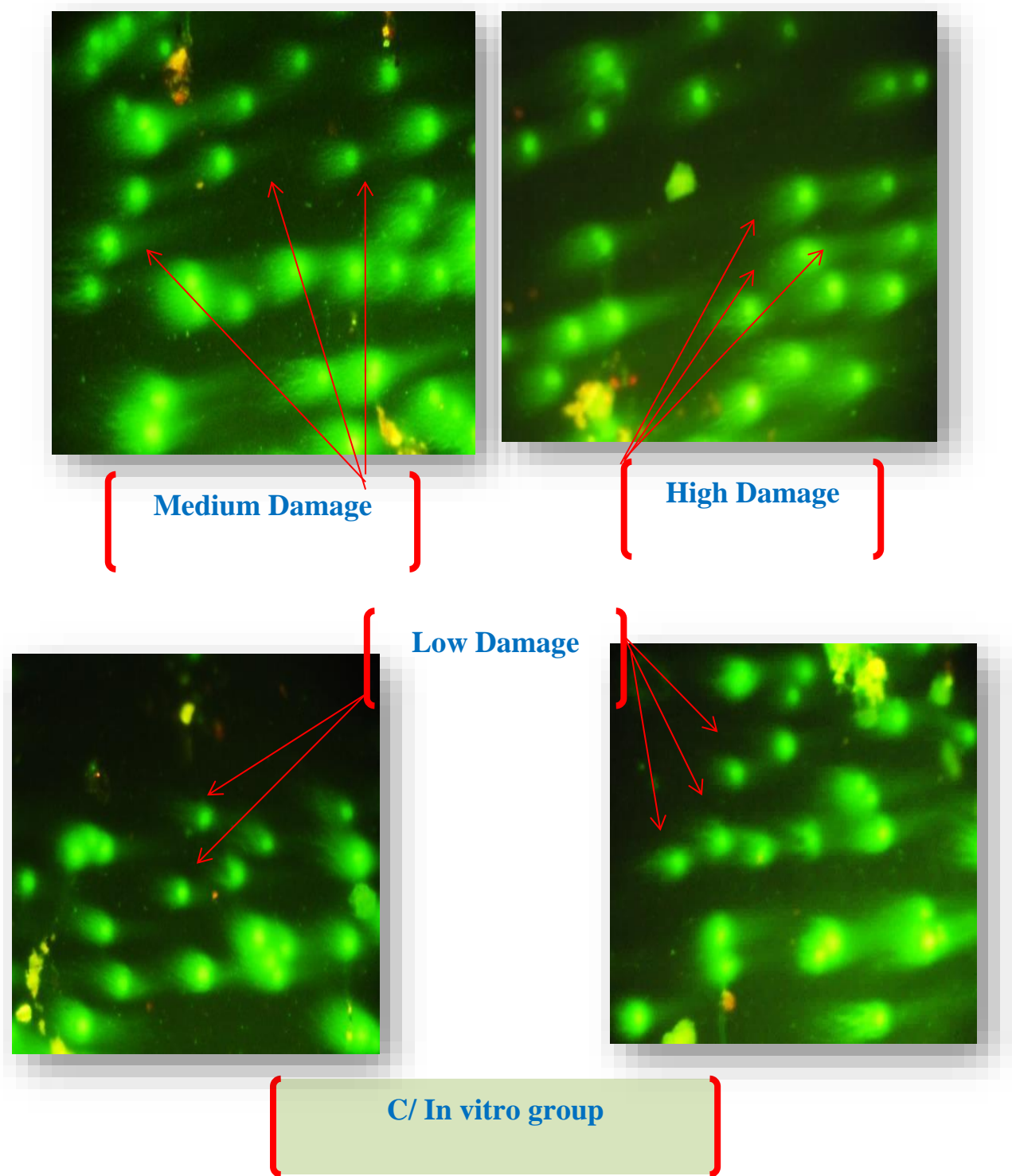


Fig 4.6. Comet photos depicting DNA movement patterns in human mesenchymal stem cells stained with Vista Green DNA Dye **A:** The non-infected control group, in which most of the cells lacked a comet. The DNA was tightly compressed and maintained the usual nucleus' circular arrangement.; **B: naturally infected;** the profile of nuclear DNA in this group was altered by the emergence of a fluorescent streak extending from the nucleus; **C: invitro infected group;** photos revealed an increase in the number of damaged DNA in MSCs.

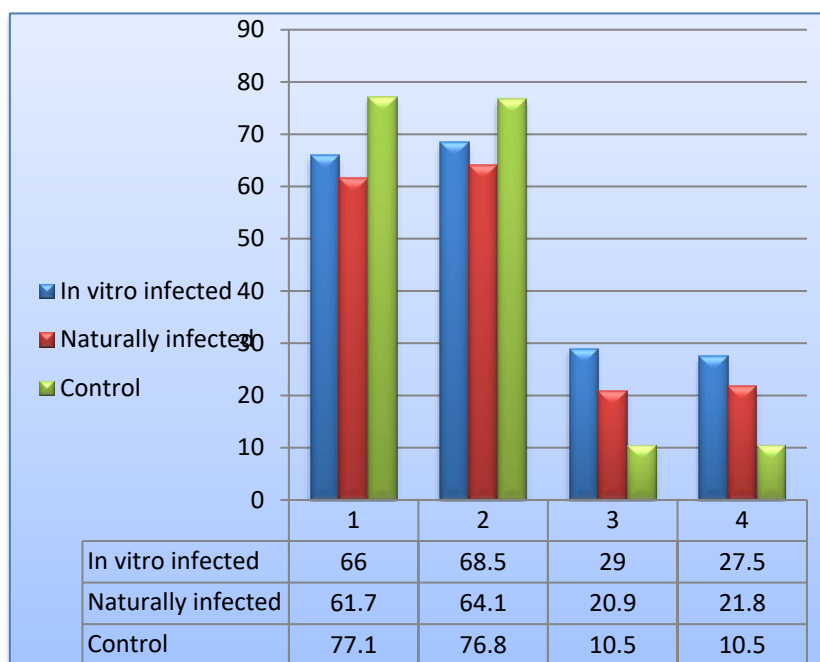


Fig 4.7. Average of DNA damage ,1/ NO damage , 2/ low damage, 3/ medium damage, 4/ high damage.

IV. DISCUSSION

Toxoplasma gondii infection requires autophagy and apoptosis to be controlled. During pregnancy, *T. gondii* infection can harm the fetus and cause birth abnormalities; however, the molecular processes underlying this process are unknown. Autophagy and apoptosis are both crucial defense mechanisms against invading pathogens in the host. In the activation of host cells to defend against intracellular infections, autophagy performs an antibacterial and antiparasitic role; yet, pathogens could take use of autophagy in the host cell to enhance their proliferation. In eukaryotes, apoptosis is required for cell growth and tissue homeostasis, and also plays a role in disease pathogenesis. [16]. Single strand breaks (SSBs), double strand breaks (DSBs), missing bases, and chemical alteration of nucleotides are all examples of DNA damage. [17].

T. gondii ROP18 kinase, on the other hand, has been shown to interact with host proteins involved in DNA repair and other activities [18]. Humans with significant polymorphisms in DNA repair genes have verified ocular and brain injuries induced by *T. gondii* infections. [19].

Our study is consistent with [20], study which confirmed that High-virulence infection Apoptosis and significant DNA damage were caused by the **T. gondii** strain, particularly when the acute infection was prolonged. DNA fragmentation and apoptotic laddering were found to be more intense in infected splenocytes and blood leukocytes, and DNA migration in the comet tail in peripheral blood was much higher than in the control group. In addition, caspase 3 immunostaining revealed a positive reaction in the infected group's splenic region after DNA fragmentation.

Our study is consistent with [21] their study confirmed In comparison to the control group, *T. gondii* infection caused a statistically significant increase in the frequency of tailed nuclei (DNA damage) in mouse retinal cells (which showed some degree of DNA damage).

For genomic stability and cell viability, maintaining DNA integrity is critical. Endogenous metabolic activities and exogenous environmental influences constantly damage the DNA, altering its chemical structure. Single strand breaks, double strand breaks, inter- and intra-strand crosslinks, and base alterations are all examples of DNA lesions. oxidation and alkylation of bases, creation of bulky chemical adducts, and crosslinking of neighboring nucleotides are only a few examples. Mutations, deletions, insertions, translocations, and loss of chromosomes and important genetic information are all possible outcomes of DNA damage. As a result of the genome instability, apoptosis and deadly illnesses can occur [22].

Parasites, such as *T. gondii*, have been shown in some studies to cause DNA damage in cell essential components. [23] Endogenous DNA damage could occur as a result of reactive oxygen species created by normal metabolic

processes, particularly during the process of oxidative deamination [24]. Cell cycle control, gene expression, and cytoplasm/mitochondrial communication are all thought to be affected by oxidative stress. [25]. The host immune response against *T. gondii* infection, which includes antibody production (humoral immune response) and activation of cellular mechanisms, may cause DNA damage in *T. gondii* infected cells. [26].

Macrophages can release nitric oxide, interferon-gamma (IFN-), tumor necrosis factor (TNF-), and reactive oxygen species (ROS) during cellular immunological responses [27]. While such processes aid in the eradication of *T. gondii*, they do so at the expense of exposing target organs to endogenous genotoxic chemicals that react directly with DNA. [28]. The degree of DNA damage observed in the control group could be explained by the fact that roughly 10000 oxidation hits to DNA per cell occur per day in the human body, and DNA in vitro contains more than 35 different types of oxidized bases. [29].

Although most DNA damage is repaired by efficient DNA-repair enzymes, some damage is not repaired, resulting in permanent damage [30]. Using the comet assay, DNA damage in peripheral blood, liver, and brain cells caused by *T. gondii* infection was observed to occur solely in leukocyte cells and not in liver and brain cells. Early infection is linked to the host response against *T. gondii* infection, which includes the cellular-immune host response. Macrophages, neutrophils, and dendritic cells have been found to release IFN-, TNF-, nitric oxide, and reactive oxygen species in response to the parasite [31].

Although these aid in *T. gondii* eradication, they expose organs to endogenous genotoxic chemicals that cause DNA damage in peripheral blood cells. Other investigations found that infection with high-virulence *T. gondii* is related with high levels of pro-inflammatory cytokines such IFN- and widespread splenic lymphocyte death, and suggested that the high levels of pro-inflammatory cytokines may promote splenic apoptosis [32].

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