ORIGINAL ARTICLE

Evaluating the Genotoxicity of Hydroxychloroquine: An *In Vitro* and *In Vivo* Study

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Received: 05 April 2024
Revised: 06 May 2024
Accepted: 07 June 2024

Abstract

Background: Hydroxychloroquine (HCQ) is a drug used to treat malarial parasites and it was extensively used during the initial phase of COVID-19. However, HCQ demonstrated certain serious effects when administered to patients. Hence, this study intends to determine its toxicity by exposing it to human peripheral blood and the fly model.

Methods: In the present experimental study, HCQ (200 mg) was tested in different volumes (62.5 µl - 500 µl) on human blood samples (*in vitro*) and *Drosophila melanogaster* (*in vivo*). Hemolytic assay, trypan blue assay, mitotic index, chromosomal aberration, and DNA fragmentation assay were used to assess the sublethal effects of HCQ.

Results: The results implied that HCQ, at its highest concentration (500 µl), showed maximum lysis in the hemolytic assay, and an increased number of dead cells were observed with increasing concentration in trypan blue assay. Also, the percent mitotic index decreased with increasing concentration of HCQ. Chromosomal aberrations, including breaks, centromeric disruption, dicentrics, and pulverized chromosomes, were observed on exposure to HCQ. The number of fragments in agarose gel electrophoresis revealed damage to DNA. Therefore, these results provide evidence and prove the cytotoxicity and genotoxicity of HCQ.

Conclusion: HCQ is found to have cytotoxic and genotoxic effects. These results imply that further examinations must be conducted before prescribing HCQ to treat various diseases.

Please cite this article as: Sankar S, Redipalli S, Jayaraj I, Ghosal S, Parvathi VD. Evaluating the Genotoxicity of Hydroxychloroquine: An *In Vitro* and *In Vivo* Study. J Health Sci Surveillance Sys. 2024;12(3):251-259.

Keywords: *Drosophila melanogaster*, Cytotoxicity, Human peripheral blood, Genotoxicity, Hydroxychloroquine

Introduction

Hydroxychloroquine (HCQ) belongs to a class of drugs known as 4-aminoquinoline, which possesses anti-autophagy, immunosuppressive, and antimalarial properties. Plaquenil (C18H26ClN3O), an analog derived from antimalarial drugs, has been extensively used in the treatment of protozoal infections caused by *P. malariae, P. falciparum, P. vivax, and P. ovale*.[1] HCQ is a solid-type medicine with a mass of 335.9 g/mol. It is slightly soluble and exhibits reasonable solubility with glucose in water or saline. Its mechanism of action includes increasing the activity of lysosomes in antigen-producing cells and inhibiting the organelle catalyst activity in parasitic cells.[2] Although the precise mechanism of action is unknown, Plaquenil may suppress immune function by interfering with the processing and presentation of antigens and the production of cytokines. As a lysosomotropic agent, Plaquenil raises the intra-lysosomal pH, impairing autophagic protein degradation. The accumulation of ineffective autophagosomes mediated by Hydroxychloroquine may result in cell death in tumor cells. This agent is active against the erythrocytic strains of *P. malariae*, *P. vivax,* and other strains of P. *falciparum*.[3]

Plaquenil also has its application as an anti-rheumatic in systemic lupus erythematosus. Plaquenil is 67-74% bioavailable, with 16-21% of its dose believed to be excreted without alteration.[4] HCQ has a half-life that extends to one month and requires six months to

be eliminated from the body. This is important when addressing side effects like tissue layer deposits, skin sensation, and retinal toxicity.[5] Long-term usage of HCQ can cause hydroxychloroquine retinopathy, in which secondary obstruction of photoreceptor cells occurs. Therefore, it is advised to take HCQ at the dosage prescribed by clinicians.[6]

HCQ is an antimalarial medicine recently used off-label extensively by front-line healthcare staff and individuals as a prophylactic drug against COVID-19 in several parts of the world.[7] On July 15, 2020, the Food and Drug Administration declared that HCQ, CQ, and their various analogs can cause abnormal heart rhythms like QT interval prolongation and *cavum arrhythmia* when combined with azithromycin and other drugs. Hence, the FDA suggested not using HCQ and CQ.[8] COVID-19 patients are immunologically susceptible due to their advanced age, comorbidities (such as polygenic disorder, fleshiness, and vessel disease), and ensuing co-medication.[9] The urinary system and liver metabolize HCQ and CQ. Also, HCQ and its various analogs have a narrow therapeutic window and increased toxicity. A one-time dose of 30 mg/kg CQ has resulted in fatalities. Internal organ side effects of HCQ include conduction disturbances (bundle-branch block, complete or incomplete arrhythmia, QT prolongation) and cardiomyopathy (hypertrophy and symptom heart failure).[10, 11] HCQ also decreased viability when treated on eight different cell lines, proving that HCQ has the potential to cause cytotoxicity.[12] HCQ also has the potential to generate oxidative DNA damage and mutation in mammalian cells *in vitro*. In particular, genotoxic effects of HCQ have been observed at clinically relevant dosages.[13]

The present study further explores the pharmacological perspective (pharmacokinetics and pharmacodynamics) of HCQ, along with its medical application in treating various diseases, including antimalarial and rheumatoid arthritis. No other research has been reported on human peripheral blood (*in vitro*) to test the effects of HCQ. The cytotoxicity and mutagenicity of HCQ in human peripheral blood were analyzed using a hemolytic assay, trypan blue assay, and mitotic index assay to re-evaluate the toxicity of HCQ. This is the first study to be conducted on an *in vivo* model to test the genotoxicity of the drug. HCQ was exposed to *Drosophila melanogaster*, and its ability to fragment DNA was tested. The choice of the fly model is further justified owing to the genetic homology it shares with humans.

Mutagenicity and Genotoxicity of HCQ

HCQ produces two first-stage metabolites, desethylhydroxychloroquine and desethylchloroquine. Desethylchloroquine is also the first-stage CQ metabolite. These first-stage metabolites are further processed to give rise to a standard product, bisdesethylchloroquine, which induces mutagenic effects in bacterial systems.

This metabolite mediates its toxicity by interacting with DNA and producing an intercalated complex that may induce frame-shift mutations. Additionally, it induces lethal recessive mutations in the sex chromosomes of *Drosophila melanogaster* and causes oxidative stress in animal models. N-nitroso compounds are formed in the gastric environment when HCQ or CQ are administered with drugs containing nitrites. Consequently, the N-nitroso compounds perpetrate genotoxic effects and induce tumor formation in animal models.[14, 15] The genotoxic effects of HCQ have not been reported in humans thus far.

Mechanism of Action of the Drug - HCQ

The primary mechanism of action associated with this drug is complex. It follows a similar mechanism to chloroquine, a blood schizonticide that impacts all Plasmodia, effectively against the erythrocytic forms of all strains of *Plasmodia*.[16]

Recent research studies have elucidated that this drug tends to accumulate inside the lysosomes found in malarial parasites and increases the pH in the vacuoles. This increase in the pH obstructs the proteolyzing activity of hemoglobin in malarial parasites, thus disrupting the mechanisms underlying the growth and replication of the parasite. It also tends to hinder the mechanism involved in haem-polymerization within the parasite. This results in the exudation of beta-haematin (a toxic product) into the parasite (Figure 1).[17, 18]

Further, HCQ also increases the pH by accumulating in various organelles found in the cytoplasm of human cells.

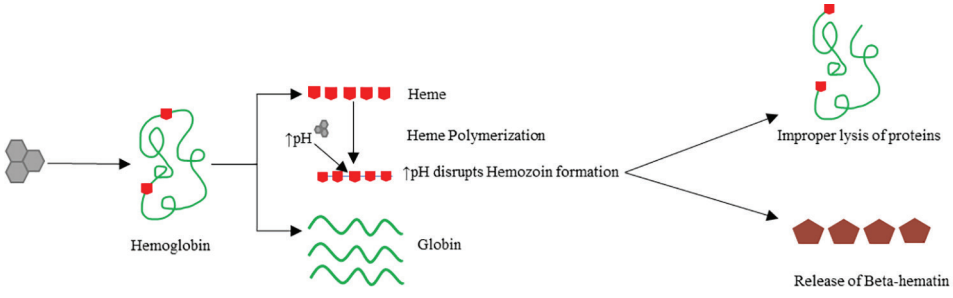

Figure 1: Mechanism of action of HCQ on *P. falciparum* (Original Diagram)

Consequently, processes like dimerization of alpha and beta chains of the MHC II complex, antigen processing, antigen presentation, and the quantum of inflammatory responses are hampered.[19, 20] HCQ tends to elevate the pH in organelles; this phenomenon is hypothesized to alter mechanisms underlying MHC recycling. As a result, only MHC complexes exhibiting high affinity are presented on the cell surface.[17, 21] Further, HCQ plays a role in inhibiting the Toll-like receptor functioning, thus blocking NOX-mediated signaling and reducing cytokine (IL-8 and TNFα) release.[12, 13] Elevated pH levels inside organelles, such as endosomes, obstruct the activity of viral components by hampering their ability to bind to the ACE-2 receptor and enter the host cells. This is mediated by terminal glycosylation of the ACE-2 (Figure 2).[22-24]

In-Silico and In-vitro Analysis of the Drug HCQ

Upon calculating the binding energies of HCQ and CQ after they bind to the ACE-2 receptor on the surface of host cells, it was discovered that HCQ exhibits an increased affinity compared to CQ towards ACE-2 (angiotensin converting enzyme-2) and Mpro (viral main protease). This can be attributed to the structural differences between HCQ and CQ. The extra hydroxyl group characteristic of the HCQ structure confers the drug with increased molecular interactions and binding properties. Furthermore, HCQ also exhibits tendencies to bind to gangliosides in the respiratory system, thus preventing SARS-CoV-2 from binding to them and causing respiratory defects.[25] Another *in-silico* study has proved that molecular docking between HCQ and SARS-CoV-2-NTD-N-protein using a semi-empirical free energy force with ten modes of interaction between the drugs results in obtaining NTD-N-protein. NTD-N protein is an RNA-binding nucleocapsid protein present in the core of a virus. NTD-N protein is present between the NTD and C terminals of the serine-associated region and plays a vital role during replication. The molecular docking process with ligands showed that HCQ and CQ have high interaction with NTD-N-Protein, which will reduce the virulence of the SARS-CoV-2 virus. Therefore, the results of the computational study confirm that the interaction between HCQ and NTD-N-protein is more than CQ.[16, 26]

Another research study on HCQ and azithromycin reveals that both drugs have active binding on Cys145 and His41, which are predominant protease enzymes and inhibitors. Hence, the combination of these two drugs substantially affected the SARS-CoV-2 virus, but its mechanism of action still needs to be identified.[27] HCQ binding to serum was insignificant, binding up to 50-60% to plasma protein. An *in vitro* study conducted *hitherto* has analyzed the interaction between the CQ drug and Vero E6 cells infected with the SARS-CoV-2 virus. The result showed that CQ possesses an antiviral activity by increasing endosomal pH. It also interferes with the cellular receptors that undergo glycosylation.[28, 29] Research studies have demonstrated that HCQ inhibits calcium signaling in immortalized human T-lymphocyte cell lines. It induces CD69 expression (60-80%) and inhibits TCR (T-cell receptor) activation. It is inferred that HCQ inhibits TCR-induced calcium mobilization between cells.[30]

A combination of HCQ and azithromycin was administered to control SARS-CoV-2 virus replication *in vitro*. This is due to the action of azithromycin, which perfectly binds with hydroxychloroquine, and the coupling of ions such as Mg2+, Ca2+, or Zn2+ occurs, forming a complex structure.

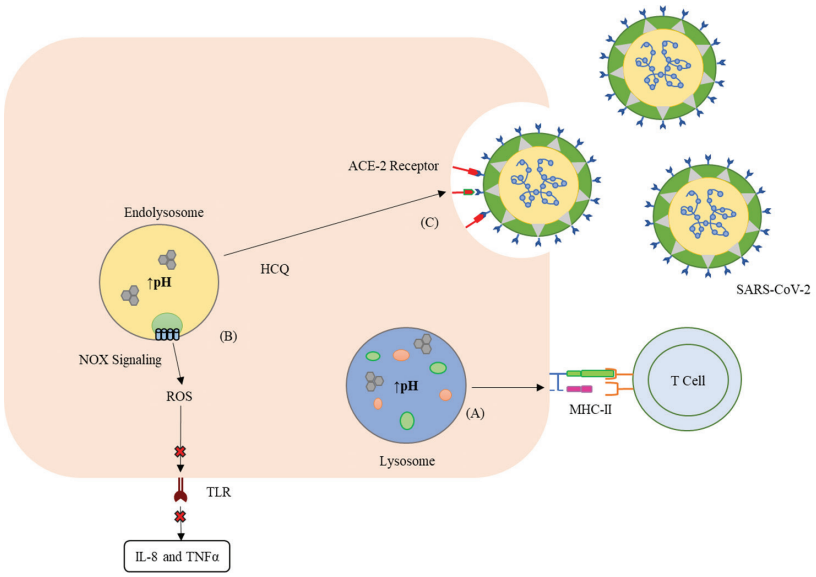

Figure 2: Mechanism of action of HCQ on animal cell (Original Diagram). A. HCQ elevates pH in various organelles and disrupts the MHC Process. B. HCQ inhibits Toll-Like Receptor (TLR) by blocking NOX signaling and thus inhibiting cytokine release. C. The binding of the ACE-2 receptor with SARS-COV-2 spike protein gets hampered due to elevated pH levels in endolysosome.

Also, HCQ contains two aromatic rings with Cl end and couples with azithromycin. Moreover, the open NH region of HCQ combines with the end of the Cl group and creates a coupling with the uracil nucleotide. Azithromycin blocks the temporary binding between the OH end of HCQ and the adenine uracil nucleotide until it binds to the uracil nucleotide. The rotation of the Mg2+ complex of azithromycin and HCQ couples with RNA pairing of adenine-uracil and thus inhibits translation and replication of SARS-CoV-2.[31] Research studies have proved that HCQ stimulates apoptosis, inhibits cancer cell growth, and reduces phosphor S6 levels in RCC cell lines. The combination of HCQ and tamoxifen (TAM) inhibits the growth of LCC9 xenograft cells in treating ER+ breast cancer.[32, 33] In another study, WBC taken from patients with systemic lupus erythematosus (SLE) were cultured and treated with HCQ. After the treatment with HCQ, cells' viability decreased, and apoptosis was demonstrated. Accelerated apoptosis has been observed in WBC when treated with HCQ rather than average. This showed that HCQ induced apoptosis.[34]

Methods

Preparation and Exposure

Hydroxychloroquine (200mg) from IPCA Laboratories was dissolved in 300ml of 5% dextrose solution prepared in sterile distilled water, resulting in a 1mg/ml concentration. Four volumes of HCQ (62.5 µl, 125 µl, 250 µl, 500 µl) were exposed to a human blood sample and *Drosophila melanogaster*. Benzaldehyde (5 µl) (#37278, SRL Chemicals) was used as a positive control.

Haemolytic Assay

Human peripheral blood (5 ml) was collected in a heparin tube and centrifuged at 1000rpm for 10 minutes. A 1% RBC suspension was prepared. 200µL of the suspension was tested for working concentrations and controls. The contents were incubated for 2 hours and then centrifuged at 1000 rpm for 10 minutes. The supernatant was loaded into a 96-well flat-bottom plate, and its absorbance was measured spectroscopically at 545 nm. The percentage of hemolysis was calculated using the following formula:[35]

$$\text{Percentage of hemolysis} = \frac{\text{OD of the test sample} - \text{OD of the negative control}}{\text{OD of the positive control} - \text{OD of the negative control}} \times 100$$

Trypan Blue Assay

Five milliliters of blood were collected in an EDTA tube and layered on an equal volume of Ficoll Histopaque. The sample was centrifuged at 2500rpm for 30 minutes, and the buffy coat was isolated. The cell suspension was washed with PBS, and a 1% WBC suspension was prepared. 100µl of WBC suspension was transferred into a hemocytometer to perform cell counting. 1.2×10^8 WBC cells were transferred and incubated in a 96-well flat-bottom plate. 500µl of the suspension was tested for working concentrations and controls. After 2 hours, 25 µl of the suspension was taken and mixed with 100µl of trypan blue. Live and dead cells were counted using a hemocytometer, and viability was calculated using the following formula:[35]

$$\text{Viability of cells} = \frac{\text{No. of live cells}}{\text{No. of live cells} + \text{No. of dead cells}} \times 100$$

Mitotic Index Assay

One milliliter of heparinized whole blood was cultured in 8ml of RPMI 1640 (#AL028A, Himedia) and 2 ml of FBS in a culture flask. 400µl of PHA was added and incubated at 37ºC. After 66.5 hours of incubation, 100 µl of EtBr was added to each flask and incubated for 30 minutes. At the 67th hour,100µl of colchicine was added and incubated for another hour. Post colchicine treatment, the cells were treated with 0.075M KCl as the hypotonic solution and incubated at room temperature for 20 minutes. Cells were centrifuged at 1000 rpm for 10 minutes, and the pellet was fixed with 3:1 methanol and acetic acid (Carnoy's fixative). The pellet was washed several times with the fixative, cast on clean, grease-free glass slides, and stained with Giemsa (#S011, Himedia). Mitotic index and chromosomal aberrations were observed and documented under 100× magnification.[36]

Isolation of DNA from Drosophila

Canton-S (30 flies per experiment group in duplicates) were exposed to test concentrations and control for 48 hours. Post-exposure, flies were crushed with 500µl of solution A (Tris HCL-100mM, EDTA-100mM, NaCl-100mM, SDS-0.5%) and incubated on ice for 15 minutes, followed by treatment in a water bath for 30 minutes. The contents were centrifuged at 13000 rpm for 15 minutes. Phenol chloroform (#MB078, Himedia) of equal volume was added to the supernatant and centrifuged at 13000 rpm for 5 minutes. To the supernatant, 150µl of isopropyl alcohol was added and centrifuged at 10000 rpm for 5 minutes. To the pellet, 1 ml of 70% ethanol was added and centrifuged for 5 minutes at 13000 rpm. The pellet was air-dried overnight, resuspended in 100 µl of 1×TE buffer (#ML012, Himedia), and stored at -20ºC.[37]

DNA Fragmentation Assay by Agarose Gel Electrophoresis

The isolated DNA samples were run on a gel electrophoresis apparatus using 1.5% agarose gel (#MB002, Himedia) and TAE buffer (#ML016, Himedia). 8 µl of EtBr was added, and the gel was

allowed to solidify. To 8 µl of the sample, 2 µl of 6X gel loading buffer (#ML015, Himedia) was added in the wells with a 100bp DNA ladder (#MBT049, Himedia), and the bands were visualized under a UV trans-illuminator to check the fragmentation of DNA.[34]

Results

Increase in Hemolysis

The hemolytic assay demonstrated that HCQ induces hemolysis in red blood cells (RBCs). An increase in HCQ volume significantly increased hemolysis. Specifically, hemolysis percentages of 5.37, 12.83, 16.41, and 21.49 were noted in T1, T2, T3, and T4, respectively (Figure 3).

Decrease in Cell Viability

As the volume of hydroxychloroquine (HCQ) increased, a decrease in the viability of white blood cells (WBCs) was observed. The recorded viability of WBCs was 80.88%, 72.41%, 67.85%, and 63.15% for T1, T2, T3, and T4, respectively (Figure 4). Notably, more dead cells were observed in T4, with values approaching those of the positive control.

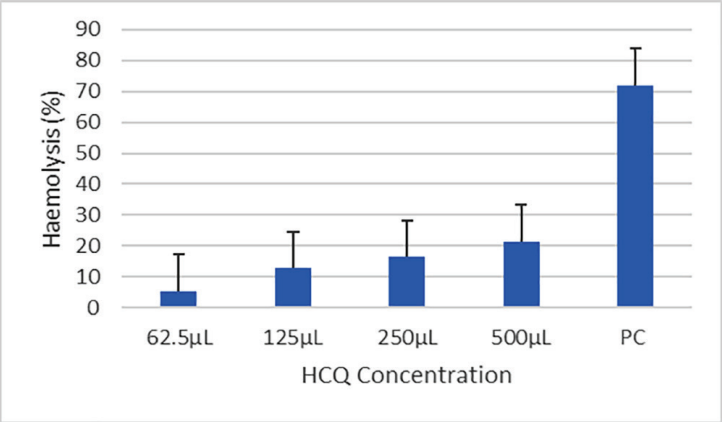

Figure 3: Percentage of hemolysis observed in RBCs after exposure to HCQ drug

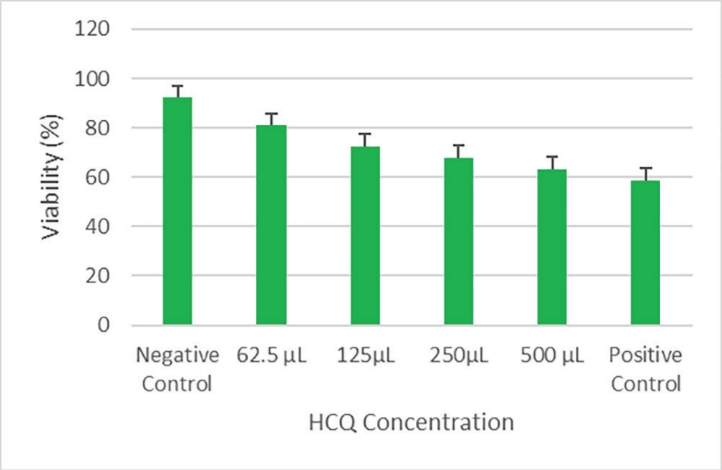

Figure 4: Percentage of viability in WBCs after exposure to HCQ drug.

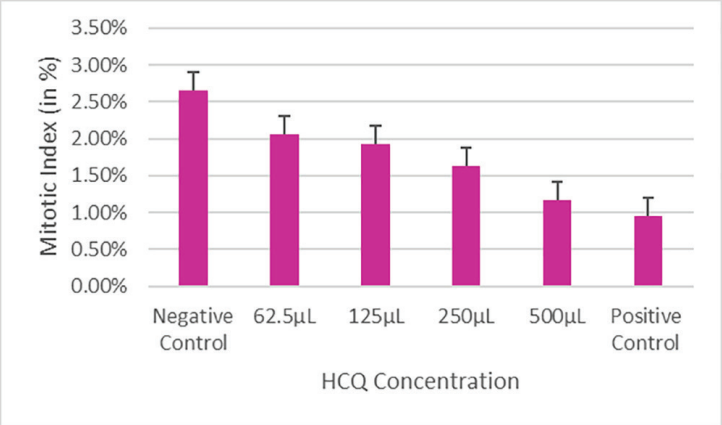

Figure 5: Percentage of mitotic index observed in cells after exposure to HCQ

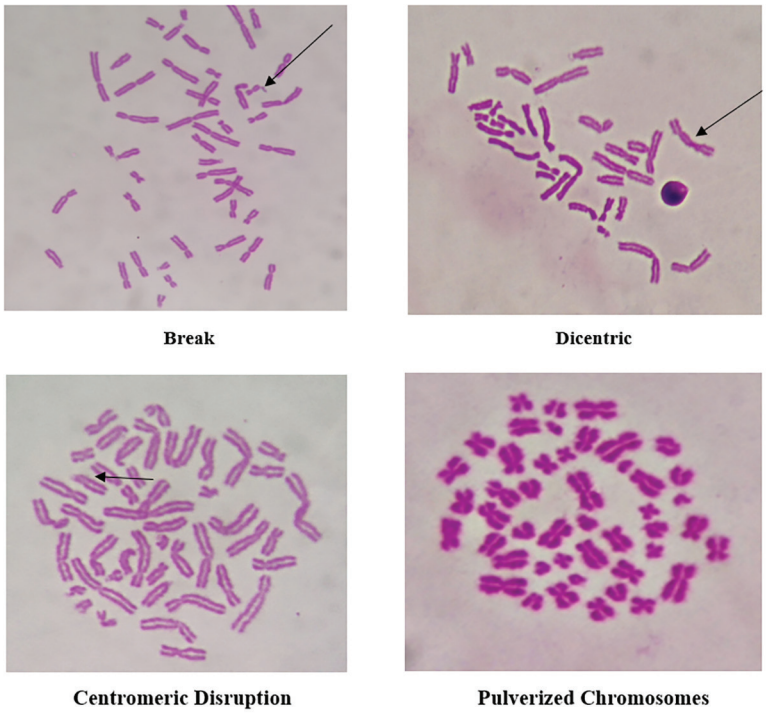

Figure 6: The above images represent the abnormal chromosomal aberrations (Original Picture)

Decrease in Mitotic Index

The mitotic index percentages of blood white blood cells (WBCs) decreased in response to an increase in hydroxychloroquine (HCQ) concentration. Specifically, with 29 metaphase cells in T1, a mitotic index of 2.06% was observed. This decreased to 1.92% in T2, 1.63% in T3, and 1.17% in T4, respectively. The number of interphase and metaphase cells decreased with increased volume. These results suggest that the highest concentration of HCQ corresponds to the lowest mitotic index (Figure 5).

Chromosomal Aberrations

Abnormal chromosomal aberrations were observed in each sample concentration under 100× magnification. An increase in chromosomal aberrations was noted at the highest concentration. The observed chromosomal aberrations included inter-chromosomal breaks, dicentric chromosomes, centromeric rupture, and pulverized chromosomes (Figure 6).

DNA Fragmentation Assay

Bands of fragmented DNA were detected in agarose gel electrophoresis. As the volume of HCQ increased, thicker bands were observed. These thick bands in the test samples typically indicate increased DNA breaks and damage. The well with the highest volume, T4, showed the most fragmentation, followed by T3, T2, and T1 (Figure 7). The bands were compared with positive and negative controls, using a DNA ladder as a reference. The treatment with HCQ significantly induced apoptosis and DNA damage, as evidenced by the fragmentation bands.[34]

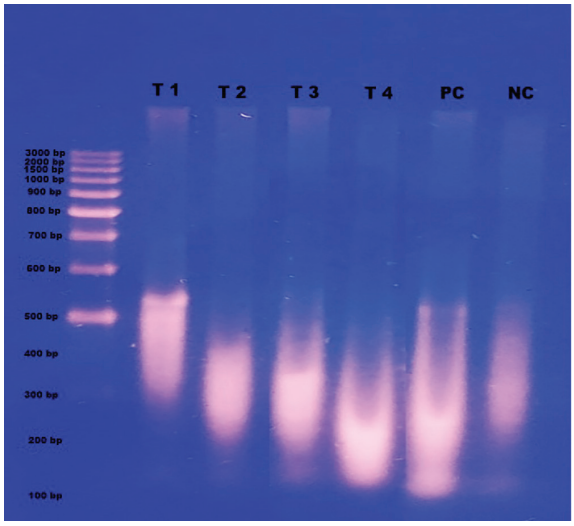

Figure 7: The above image represents the DNA Fragmentation of HCQ (Original Picture)

Discussion

Ever since HCQ has been used to treat diseases like malaria, rheumatoid arthritis, and lupus, many side effects have been reported, studied, and documented following its consumption. Noteworthy side effects include Ocular toxicity[5], cardiac effects[8], and neurological problems.[9, 38] Initially, HCQ was used to treat COVID-19 patients due to its antiviral properties. However, HCQ administration was later withdrawn due to its prolonged side effects and limited effects on the SARS-COV-2 virus.[8, 11] Only limited data is available to study the cytotoxic effects of HCQ on *in vitro* mammalian cells.[13] Further, no research has been conducted to support HCQ's genotoxic effects

on the *in vivo* model. Hence, it is inevitable to conduct further research on a functional mammalian *in vivo* model to obtain data about these similar effects of HCQ.

Adverse side effects of HCQ were observed in both human peripheral blood and *Drosophila melanogaster*. An increase in hemolysis following HCQ exposure suggests that HCQ induces erythrocyte disruption. HCQ enters erythrocytes to produce free radicals and interrupts the hemoglobin mechanism, which in turn causes hemolysis. Similar hemolytic effects have been observed in glucose-6-phosphate dehydrogenase deficiency (G6PD) patients when introduced to HCQ.[39]

Trypan blue assay is used to analyze dead or damaged cells. A decrease in cell viability was observed when exposed to the drug, adding that HCQ induces loss of viability in lymphocytes. The number of dead cells increased with the increased HCQ exposure to the WBC cells. HCQ, accumulated inside the WBC cells, increases lysosome activity and pH, obstructs cell functions, and leads to death. The same inhibitory effects of HCQ on various cell lines were observed through a live cell analysis system.[12] It explains that HCQ induces inhibitory effects on cell growth and proliferation, which leads to decreased cell viability. A decrease in the mitotic index shows the interference of HCQ in the cell cycle, which inhibits white blood cells from reaching metaphase. It also indicated that cells do not actively participate in cell division. This proves that HCQ has a cytotoxic potential to hinder cell division and replication. Chromosomal aberrations show that HCQ can penetrate the nucleus, interact with, and damage the chromosomes during cell division. The presence of dicentric chromosomes and centromeric disruption indicates that exposure to HCQ leads to genetic instability. Chromosomal breaks suggest the drug has caused chromosome fragmentation and its potential mutagenic effect. The intensity of DNA fragmentation provides information on the dose-dependent effect of HCQ. Similar DNA damage was also observed in mammalian cells by quantifying 8-oxodG levels after exposure to the drug. It is clear from the chromosomal changes and DNA fragments that exposure to HCQ resulted in genomic instability.

Conclusion

Although this novel research advances our understanding of the cytotoxicity and genotoxicity of HCQ in both *in vitro* and *in vivo* models, there are a few limitations. Further investigation of HCQ's genotoxicity in mammalian models is needed to provide more comprehensive results. Given that HCQ has been linked to QT interval prolongation, retinal toxicity, and neurological issues, it is essential to conduct organ-specific toxicity tests, focusing on the heart, brain, eyes, liver, and kidney. The DNA damage and abnormal chromosomal aberrations reported in this study can be further used to evaluate the relationship between HCQ and genes associated with DNA damage.

In summary, our results substantiated that HCQ has the potential to cause cytotoxicity and genotoxicity, as assessed in both *in vitro* and *in vivo* models. Our findings challenge the conclusions drawn from similar studies conducted to evaluate the toxicity of HCQ[12,13]. The cytotoxic and genotoxic effects of HCQ that we observed lead us to question its use for various diseases and underscore the need for further evaluation.

Acknowledgment

This research work was partially funded by the Summer Research Fellowship 2021, awarded to Ms. Sharmishta R by Sri Ramachandra Institute of Higher Education and Research.

Conflict of Interest: None declared.

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